# Breaking the Parasite lifecycle

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A thesis

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by

Marsha Andrea Martin-Mckie

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Abstract of a thesis submitted in partial fulfilment of the
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This thesis investigates the use of either liquid urea or effective microorganism solutions to break the parasite lifecycle while outside of its host and reduced larval challenge on pasture. The main aim was to extend *in vitro* results of urea and Effective micoorganisms on egg development in the field. The field study was performed in a replicated randomized block design at the Lincoln University Lincoln Sheep Research Farm from February 9th 2016 to April 22,2016 on 0.71 ha of newly sown rye grass (*Lolium perenne*) and white clover (*Trifolium repens*) pasture. Five-month-old Romney lambs that were naturally infected with gastrointestinal parasites through grazing contaminated pastures and which had not received anthelmintic treatment for a minimum of six weeks rotationally grazed the areas to seed the pasture with fresh contamination for two-days in each plot before being moved to an adjacent plot, giving eight replicates of treatment across time. Immediately upon the removal of lambs from each plot, the areas were topically sprayed with the equivalent of 200 litres per ha of either flowfert N (Ravensdown Ltd) (40 units per ha), effective microorganism mixture (EM; Nature farm Ltd) or water ($H_2O$) with each treatment replicated twice within each time, giving 16 replicates in total. Following seeding of the pasture with contamination, lambs were given anthelmintic and allowed to graze worm free pastures for seven days. Lambs were weighed and allocated to one of six groups of five animals that were balanced for live weight and then allowed to graze a treatment, grazing each area of the same treatment for two days. Variables such as pasture larval contamination and pasture mass was recorded immediately prior to grazing on each plot with post-grazing pasture mass also recorded. Animal performance and faecal egg count measurements were recorded weekly. Further, on one day of treatment, six trays each of which contained 100 grams of fresh sheep faeces averaging 800 eggs per gram were placed on respective treatment plots, they were sprayed with the respective treatment then left overnight before being taken to the laboratory and cultured for 10 days at 25 °C. In addition, a second *in vitro* larvae culture was performed where the culturing conditions of with and without a plastic bag were compared following the topical application of urea and water to see how each treatment perform under the same environment.

Overall, both Effective microorganism (EM) solutions and urea had little influence on breaking the parasite lifecycle when applied in the field. For pasture larval contamination, lamb FEC, LW and DMI, there were no significant difference. This may reflect the design of the field study which the two days rotation provided an opportunity for half of the eggs to hatch. *In vitro* results suggested urea has a very potent effect on egg development with 98 % reduction. In the field, urea may have inadvertently encouraged *Nematodirus* development, which may either be from increased irrigation of 12 h, or the amount of urea that penetrated the faecal mass, may not have being sufficient when dealing with faeces of different moisture or different surface area as well as pH, which was not measured in the field. In addition, *in vitro* laboratory results, with plastic bag and field study shows little development in egg hatching. Although the disparity is not clearly understood between the field and the *in vitro* results, the possibility of an artificial high ammonia concentration was ruled out. Overall, urea may provide an opportunity to break the parasite lifecycle, but further investigations are needed in the field.

For the EM treatment, *in vitro* results showed an increase of 2.5-fold in egg development which did not different from the field trial. Further investigation may be worthwhile to see if treatments of EM can be designed to stimulate larval development when survival on pasture is low at times of the year and non-susceptible stock can be grazed to effectively reduce contamination.

**Keywords:** nematode; epidemiology; larval development; Ovine; parasite control

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# List of abbreviations

| | |
|---|---|
| ANOVA | Analysis of variance |
| BOD | Biological oxygen demand |
| $CO_2$ | Carbon dioxide |
| CM | Centimetre |
| COD | Chemical oxygen demand |
| ◦C | Degree Celsius |
| DM | Dry matter |
| DMI | Dry matter intake |
| EPG | Egg per gram |
| EM | Effective microorganism |
| FEC | Faecal egg count |
| FCR | Feed conversion ratio |
| ~ | Greek Perispomeni |
| G | Grams |
| GIN | Gastrointestinal nematode |
| GLWG | Gram live weight gain |
| > | Greater than |
| Ha | Hectare |
| $H_2O$ | Water |
| H | Hour |
| i.e. | That is |
| Kg | Kilogram |
| KgDM | Kilogram dry matter |
| LW | Live weight |
| LWG | Live weight gain |
| $L_1$ | First stage larvae |
| $L_2$ | Second stage larvae |
| $L_3$ | Third stage larvae |
| $L_4$ | Fourth stage larvae |
| $L_5$ | Fifth stage larvae |
| ME | Metabolisable energy |
| Mg/L | Milligram per litre |
| Mg | Milligram |
| MJME/KGDM | Mega Joule Metabolisable Energy per Kilogram Dry Matter |

| | |
|---|---|
| ML | Millilitre |
| N | Nitrogen |
| $NH_3$ | Ammonia |
| OMD | Organic Matter Digestibility |
| PPM | Parts per million |
| / | Per |
| ± | Plus, or minus |
| % | Percentage |
| pH | Power of hydrogen |
| $r^2$ | Coefficient of determination |
| s.e.m | Standard error of the mean |
| spp | specie |
| UV | Ultra Violet |
| VS | Versus |
| Viz | That is or namely |

# Chapter 1 **Introduction**

Parasitism is a major cause of lowered production and productivity in livestock globally, especially in small ruminants. The lowered production and productivity is caused by a reduction in feed intake and feed conversion efficiency which are directly proportional to the size of the larval challenge (Coop *et al.,* 1982), thus leading to economic loss, and has further implications for both public health and food security (Van Houtert, 1997).

The cost for treating sheep and cattle with anthelmintic in New Zealand annually has been estimated to be NZ $59 million (McKenna, 1997), globally it was reported to be USD 3 billion and in the United Kingdom alone was GBP 84 million (Jackson *et al.,* 2009). Parasitism is currently controlled by anthelmintic which are losing effectiveness through gradual, but inevitable, development of resistance within the parasite population. Maintaining animal performance in the face of anthelmintic resistance requires novel and sustainable approaches to control nematode epidemiology and biology (Leathwick *et al.,* 1992; Waller, 2003). Most treatment regimes focus on treating the parasite population within the host, targeting the parasite when outside the host may be an alternative solution.

Ninety percent of the parasite population on New Zealand farms may exist outside its host, depending on time of the year and environmental conditions (Familton and McAnulty, 1997). In Australia few as 3% of the *Haemonchus* parasite is harboured by sheep (Familton and McAnulty, 1997) as such, targeting this population and affecting parasite development outside of the host may be an attractive option and provide an additional tool to aid parasite control. Development for both human (Fidjeland *et al., 2016*; Pecson *et al.,* 2007; Pecson and Nelson, 2005) and sheep (Howell *et al.,* 1999) parasites has been shown to be reduced in the presence of both Nitrogen fertilizers while parasite egg hatching is also reduced in acidic conditions. Furthermore, *in vitro* studies have shown both liquid urea solution and Effective micro-organism (EM) solutions to almost completely inhibit egg hatching for both *Teladorsagia circumcincta* and *Trichostrongylus colubriformis* (Cairns *et al.,* 2017; Lewis, 2013 honours dissertation, Lincoln University). However, while *in vitro* studies indicate the application of either of these products may have the potential to break the parasite lifecycle through inhibiting development, validation in the field is still required.

### 1.1 Aim of the study

The major aim of this research was to extend the *in vitro* laboratory results done on *Teladorsagia circumcincta* and *Trichostrongylus colubriformis* by Cairns *et al.* (2017) on the ability of liquid urea solution (N) and by Lewis (2013) on Effective microorganism solution (EM). This will be further investigated in the field by topical application to fresh faeces to effectively interrupt the parasite lifecycle.

### 1.2 Objectives:

- Determine the effectiveness of application of liquid Urea or EM *in vitro*
- Evaluate the impact of treatment to break the parasite lifecycle in grazing lambs.

### 1.3 Hypothesis of Study

It was hypothesised that topical application would reduce larval development and thus provide an epidemiological and production advantage for lambs grazing pastures that were naturally infected with gastrointestinal nematode parasites.

### 2.1.1 Impacts of parasitism

Gastrointestinal nematode parasites cause loss in production in the young lamb. Infection causes reduced live weight gain, faecal scouring, reduced wool growth, reduced nutrient utilization and increased endogenous N loss. Although the extent of production loss is directly proportional to larval challenge, some variability between species exists. Steel *et al.* (1980) observed reductions of 51% and 65% in live weight (LW) gain in lambs infected with 3000 and 9500 *T*. *colubriformis* $L_3$ larvae per week, respectively. In comparison, infections with *Ostertagia (Teladorsagia) circumcincta* of 37,500 and 120,000 $L_3$ larvae per week resulted in reductions in LW gain of 37% and 53% respectively (Symons *et al.,* 1981). Moreover, Coop *et al.* (1982) reported lambs receiving 1000, 3000 and 5000 *T. colubriformis* $L_3$ larvae per day had live weight gains that were 90%, 75% and 53% respectively, of the uninfected control with only a small proportion of this loss recovered when lambs were treated with anthelmintic every 21 days. This suggests that larval challenge or the developing larvae rather than adult worms, *per se*, was the major cause of production loss. Furthermore, an additional 17 days to reach the required slaughter weight has been reported in lambs treated with an anthelmintic with a low efficacy (Miller *et al.,* 2012). Therefore, in the context of providing effective parasite control, reducing larval challenge will help reduce the impact of parasitism on livestock production.

### 2.1.2 Parasite epidemiology and larval challenge on pasture

Parasite larval challenge on pasture can vary, with larvae per kilogram of dry matter ranging from 0-30,000, and is dependent on the growth of pasture, as fast-growing pasture reduces the larval concentration (Familton and McAnulty, 1997). Larval contamination on pasture varies from year to year and typically has two peaks, Spring and Autumn (Vlassoff, 1973). In ewes, their immune response temporarily declines during late pregnancy and early lactation, known as the peri-parturient relaxation in immunity, and results in ewes becoming susceptible to nematode infection (Vlassoff, 1973).This gives rise to a small peak in pasture contamination in the spring which are then consumed by the lamb (Brunsdon, 1971; Vlassoff, 1973; Vlassoff *et al*., 2001).

Young lambs are more vulnerable to infections than adult sheep due to their lack of an effective immune response (McKenna, 1981). Lambs that remain on pasture are then subsequently exposed to larvae developed from eggs deposited by ewes in the spring and then subsequent generations which are multiplied through the lambs themselves (Brunsdon, 1963 ; Vlassoff, 1973, 1976). Pasture infection levels then typically decline in mid (January-February) when the temperature increases, peak in the autumn when warmth and available moisture are abundant and then decline in winter as larval development slows and grazing lambs begin to acquire an effective immune response (Brunsdon, 1963; Vlassoff, 1973, 1976).

### 2.1.3 Distribution of *Trichostrongylus colubriformis* and *Teladorsagia circumcincta* on New Zealand Pastures

In the South Island and Southern North Island, *Trichostrongylus colubriformis* and *T. circumcincta*, are the predominant species present, and can be found mainly in the abomasum and small intestine of animal (Charleston, 1982). *Trichostrongylus colubriformis* is a threat to young lambs 5-6 months old and is seldom seen in lambs 6-9 months of age (Tetley, 1934).

*Teladorsagia circumcincta*, causes damage to the acid producing cells of the abomasum, resulting in interference of protein digestion due to increased endogenous losses and increase pH of the abomasal fluid (Familton and McAnulty, 1997). On pasture they can be found from early June onwards (Crofton, 1957) and are threats to young lambs 3-6 months of age (Tetley, 1934).

### 2.1.4 Distribution of *Nematodirus* species on New Zealand Pastures

*Nematodirus* species are found in the small intestines of ruminants being a threat in young lambs up to 6 months-of-age (Charleston, 1982; Tetley, 1935). On New Zealand pastures they can survive extremely low temperatures and can be commonly found in the South Island (Charleston, 1982). Their lifecycle slightly differs from the other *trichostrongylids* where development to the infective stage occurs within the egg and is slower, requiring periods of chilling before hatching may occur (Charleston, 1982). There are two species of *Nematodirus* of relevance in New Zealand, they are *N. spathiger* and *N. filicollis.* Both species develop at different rates, being more rapid in *N. spathiger* than *N. filicollis* (Brunsdon, 1963; Charleston, 1982) and can be easily differentiated by their egg shell. For *N. filicollis, its* eggshell is oval and is about .0003 centimetre in thickness to the side, while for N. *spathiger* its eggshell is about .0003 to .0004 cm thicker at the sides, .0005 to .0008 cm at the poles and the shell is more pointed at the poles (Tetley, 1935). They are present on pastures in three peaks, the smaller peak occurs in spring and the two larger peaks in summer and autumn February & March (Brunsdon, 1960, 1963).

## 2.2 Lifecycle of Gastrointestinal Nematode

Gastrointestinal nematode parasites mostly share a similar direct lifecycle (Gordon, 1948) with the exception of *Nematodirus spp* (Figure 2.1) (Charleston, 1982). Sexual reproduction occurs between the adults in the lumen of the alimentary tract of the host with the resultant eggs laid by the female then excreted in the faeces by its host. The eggs remain inside the faeces where they are protected from desiccation. Under optimum conditions the eggs hatch within 15 to 20 hours to the first stage larvae $L_1$ but this differs between species (Gordon, 1948). The $L_1$ larvae feed off microorganisms within the faecal pat until shedding its cuticle and becoming the second stage larvae ($L_2$). The $L_2$ larvae continue to feed within the faecal pat until reaching the $L_3$ stage at which point the cuticle is retain and serves as protection against environmental influences and provides resistance to

desiccation (Familton and McAnulty, 1997; Gordon, 1948). The $L_3$ larvae does not feed due to the retained cuticle utilizing its body reserves to survive for periods of up to 12 months (Familton and McAnulty, 1997). In the field, development rates vary between species and climatic conditions, with development to the $L_3$ stage taking 2-3 weeks or more (Charleston, 1982). Under favourable conditions when moisture is adequate the $L_3$ then migrates from the faeces onto herbage via water films, before being ingested by it hosts (Familton and McAnulty, 1997; Gordon, 1948).

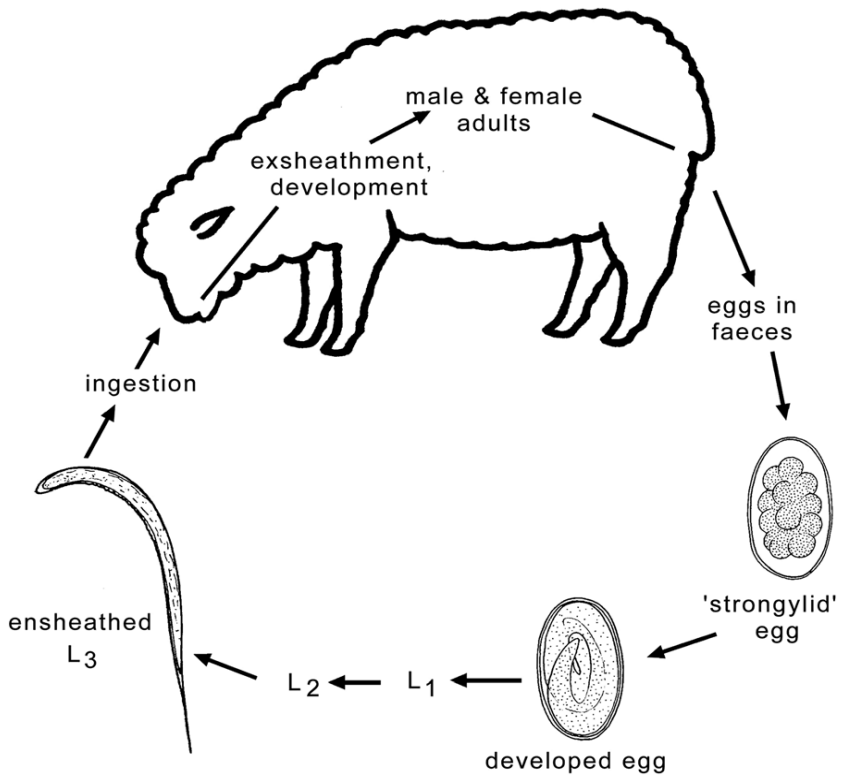


**Figure 2.1 The Life cycle representing gastrointestinal nematodes of small ruminants** (Roeber *et al*.,2013)

After ingestion by a definitive host, the $L_3$ larvae then exsheath in the digestive tract of it host in response to changes in carbon dioxide ($CO_2$), temperature and pH before reaching the infective site (Familton and McAnulty, 1997). Once reaching the infective site the larvae develop into $L_4$ then borrows into the mucosal crypt. While inside the mucosal crypt it takes a period of 8 to 10 days before the $L_4$ larvae moults into $L_5$ which becomes sexually mature adults over a further period of 7 to 10 days. Eggs from ingested larvae are then typically present (17 to 21) days following ingestion (Charleston, 1982).

### 2.3 Factors Influencing Nematode Development and survival

#### 2.3.1 Temperature and nematode development

In New Zealand, seasonal variation throughout the year in different regions favours the development of most gastrointestinal nematode (Vlassoff, 1982). On New Zealand pastures, nematode larvae are present throughout the year regardless of the different regions, with most regions experience dry warm summer and wet cool winters (Vlassoff, 1982). Development of larvae on pasture is temperature dependent, with little development occurring below 5°C and above 35°C, while optimum development occurs between 15 to 30°C depending on the parasite specie (Familton and McAnulty, 1997; Silverman and Campbell, 1959; Vlassoff, 1982). In laboratory conditions, *Haemonchus contortus* $L_3$ larvae develop in culture at temperature of 20 to 35°C (Veglia, 1916) while optimum development of *T. circumcincta* $L_3$ larvae has been reported to occur at 16°C (Pandey *et al*., 1989) and between 8 to 15°C for *N. battus* (van Dijk and Morgan, 2008). In the field, development may take longer than in the laboratory due to diurnal temperature fluctuations. In most months, less than 1% of larvae reach the infective stage and may increase to 20-25% in late summer to early autumn when the conditions of warmth and moisture are met (Vlassoff, 1982).

#### 2.3.2 Temperature and larval survival

Larval survival is dependent on temperature and although variation between species does exist, larvae can survive for extended periods on pasture. Morgan (2011) reported 2% of *trichostrongylid spp* present on pasture after 24 hrs during high temperatures and relative humidity and the numbers then changed with time as larvae move to escape the heat from solar radiation (Vegora, 1960; Vlassoff, 1982). Regardless of the time of day, most $L_3$ larvae are found at the lower 10 cm of pasture and then return to heights of above 15 cm (Gazda *et al*., 2009) when temperature decreases (Vegora, 1960; Vlassoff, 1982). Throughout seasons most *T.circumcincta* $L_3$ larvae were present for up to 11 months on pasture but decreases in survival were observed during the summer months when temperature and humidity were at the greatest (Holasova´ *et al*., 1988). This may have resulted from anhydrobiosis, which decreases the metabolic activity of the $L_3$ larvae during low and high temperatures on pasture, allowing them to survive repeated desiccation and rehydration (Lettini and Sukhdeo, 2006). In laboratory conditions, Boag and Thomas, (1985) reported *T. colubriformis* species survived at 5°C for 708 days, decreasing to 95 days at 30°C (Table 2.1). Further, Andersen and Levine, (1968) found 7% and 10% of *T. colubriformis* $L_3$ larvae survived for 128 days during desiccation at temperature of -95°C and 35°C respectively, while for the $L_1$ and $L_2$ larvae they survived for only 8 days at 30°C, demonstrating the benefit of the retained cuticle for larval survival.

| Nematode species | Median survival times (days, ±SD) | | | | | |
|---|---|---|---|---|---|---|
| | 5 C | 10 C | 15 C | 20 C | 25 C | 30 C |
| *Dictyocaulus filaria* | 263 ± 38 | 200 ± 21 | 90 ± 11 | 55 ± 5 | 41 ± 8 | 25 ± 5 |
| *Nematodirus spathiger* | 293 ± 37 | 202 ± 18 | 156 ± 18 | 90 ± 9 | 68 ± 8 | 26 ± 6 |
| *Nematodirus filicollis* | 438 ± 41 | 340 ± 38 | 248 ± 21 | 183 ± 6 | 83 ± 5 | 48 ± 6 |
| *Nematodirus battus* | 475 ± 43 | 371 ± 15 | 314 ± 27 | 252 ± 25 | 84 ± 7 | 46 ± 8 |
| *Trichostrongylus axei* | 550 ± 80 | 490 ± 54 | 366 ± 31 | 210 ± 31 | 140 ± 13 | 78 ± 9 |
| *Trichostrongylus colubriformis* | 708 ± 75 | 602 ± 67 | 525 ± 57 | 383 ± 43 | 160 ± 43 | 95 ± 12 |
| *Trichostrongylus retortaeformis* | 315 ± 29 | 260 ± 19 | 182 ± 18 | 97 ± 10 | 45 ± 7 | 31 ± 3 |
| *Cooperia curticei* | 419 ± 39 | 322 ± 29 | 243 ± 21 | 124 ± 18 | 93 ± 16 | 62 ± 13 |
| *Cooperia oncophora* | 600 ± 43 | 511 ± 41 | 430 ± 43 | 363 ± 29 | 295 ± 31 | 131 ± 10 |
| *Haemonchus contortus* | 553 ± 70 | 410 ± 36 | 392 ± 28 | 227 ± 30 | 181 ± 15 | 91 ± 10 |
| *Ostertagia circumcincta* | 825 ± 73 | 625 ± 58 | 526 ± 43 | 401 ± 30 | 304 ± 28 | 154 ± 16 |
| *Ostertagia ostertagi* | 1,133 ± 102 | 830 ± 70 | 637 ± 43 | 489 ± 41 | 381 ± 38 | 188 ± 23 |

**Table 2.1 Relationship between temperature and median survival times of the infective larvae**

Source: Boag and Thomas, (1985)

### 2.3.3 Moisture and oxygen and nematode development

Moisture is essential for the development of the $L_1$ and $L_2$ larvae which are regarded as aquatic and requires small water films, which can be in the form of rainfall, evaporation, dew and precipitation for migration to the pasture sward (Familton and McAnulty, 1997). In the field especially during dry summers, development to the infective stage may take longer in the faecal pat which may be due to optimal moisture and oxygen occurring at different times (Charleston, 1982; Familton and McAnulty, 1997). Studies has shown hardened faecal crust requires regular to light rainfall and humid conditions for rapid emergence of $L_3$ larvae (Wang *et al*., 2014). Similarly, Khadijah *et al*. (2013) reported rainfall and high soil moisture content after faecal deposition increase development of *H. contortus* and *T. colubriformis* $L_3$ larvae by 28%, allowing the conclusion that faecal moisture and soil moisture content have a positive linear relationship when using simulated rainfall. This was further corroborated by O'Connor *et al.* (2007) who found 78, 70 & 58% of *H. contortus* $L_3$ larvae developed in faeces and soil when respective simulated rainfall was 12, 24 and 32 mm, which increased respective faecal moisture content to 28, 36 and 43 %. On the other hand, the crust from the faeces act as protection during desiccation, by retaining moisture and allowing larvae to develop to the infective stage, then emerges into the pasture sward when moisture is available (Familton and McAnulty, 1997).

### 2.3.4 Moisture and larval survival

Moisture is essential for larval survival. When water is available larvae migrate from faeces by either active or passive means (Familton and McAnulty, 1997) and then migrate to the taller swards within the moisture films (Goldberg, 1968; Vegora, 1960). Throughout the dry season *H. contortus* infective larvae were found at the lower 2.5 cm of herbage and in the rainy season they were found at heights of 17.5-22.5 cm (Amaradasa *et al*., 2010). This presumably reflects the presence of water films which

the larvae require to migrate, with 1.27 and 0.04% *Trichostrongylid* larvae climbing wetted and unwetted grass blades, respectively (Silangwa and Todd, 1964).

**2.3.5 pH and nematode development**

There is limited information available on the influence of pH on larval development for sheep gastrointestinal nematodes. Khatun *et al*. (2013) reported that pH influences larval development with 39.5% of *H. contortus* $L_3$ larvae developing at pH 6, which then reduced to 3.3% at pH 3. These results are consistent with those of Ashad *et al*. (2013) who reported 39.4% and 4.4% of *H. contortus* $L_3$ larvae developed at pH 6.5 and 3 respectively, and no development at pH 2. Further, Misra and Ruprah (1973) and Stringfellow (1986) have reported reduced egg hatching of *H. contortus* at pH 11.5 compared with the optimum which appeared to be in the range of pH 6.5 to 8.5. In contrast, Dick and Leland (1973) reported that a weak acid pH of 6.4 to 6.9 resulted in no development for *Cooperia punctata* with most development occurring at a pH range of 7.6-8.1, suggesting that *C. punctata* development favours alkaline pH. Recently Cairns *et al*. (2017) reported little or no egg hatching of *T. colubriformis* when pH is less than 5 but unaffected at pH 6-13.

**2.3.6 pH and larval survival**

There is limited information on pH and larval survival for gastrointestinal nematodes, although there is some evidence from other species which supports the importance of pH. In sewage sludge treated with 10% lime and 85% calcium oxide, *Ascaris suum* eggs survived for 3 months at pH 12 (Eriksen *et al.*, 1996). In comparison, treatment of sewage sludge with 1% ammonium at pH 10, egg viability was reduced by 33% after 21 days with no survival after 40 days (Ghiglietti *et al*., 1997). In addition, Katakam *et al*. (2014) observed pH of 6.33-9.08 in pig slurry had no effect on egg hatching, but the addition of 2% urea reduced eggs of both *A. suum* and *A. galli* by 50% at pH 8.35-9.28 respectively. Maya *et al*. (2012) reported pH of 5.3 and 12.7 reduced helminth and non-helminth eggs when the right combination of dryness, pH, contact time and increased temperature were in place. They observed temperature of 45°C and pH 5.3 and 12.7, respectively, resulted in helminth and non-helminth larvae surviving for 6 and 90 days, respectively, when dryness was 90%.

**2.3.7 Ultra violet light and larval survival**

Larval survival on pasture is dependent on the amount of UV or sunlight radiation penetrating the faecal mass or pasture sward. Overgrazing of pasture can expose eggs and larvae to sunlight which can be deleterious for larval survival (Stewart and Douglas, 1938). This was further corroborated by Shorb (1943) who found no survival of *H. contortus* $L_2$ and $L_3$ larvae when they were placed on grass plots and bare ground in faeces during sunlight and with increased temperature in the summer. In laboratory conditions, 6 days of constant ultra violet (UV) radiation of nematode species *N. battus*, *T. circumcincta* and *H. contortus* $L_3$ larvae caused a 100-fold greater mortality in all three species (Van Dijk et al., 2009).

In addition, Schwartz and Price (2011) reported *H. contortus* $L_1$ and $L_3$ larvae that were exposed to UV radiation survived for 30 hours and 2 weeks respectively, indicating the sensitivity to UV is dependent on larval stage.

### 2.4 Measuring parasites and parasitism

#### 2.4.1 Faecal Egg count

Diagnosis of gastrointestinal parasitism is often undertaken using a faecal egg count (FEC), which is a determination of the concentration of nematode eggs per gram of fresh faeces. The main aim of a FEC is for the estimation of the number of worm eggs present in the faeces, while monitoring the efficacy for anthelmintic treatment to aid decision making (McKenna, 1977). The concentration of eggs recorded is influenced by faecal volume (Greer and Sykes, 2012) (Table 2.2), which is a consequence of the types, quantity and quality of forage consumed (Chaves *et al*., 2006; John and Ulyatt, 1987). The ability of a faecal egg count to reflect the worm burden of the host is also affected by variations in fecundity between parasite species. For *H. contortus*, estimates of the daily egg production per female worm are between 4000 and 10,000 eggs per day (Coyne *et al*., 1991; Gordon, 1967). By comparison, *T. colubriformis* is less fecund, being 904 to 930 eggs per female per day, while *T. circumcincta* females produce 254 and 267 eggs per day (Mupeyo *et al*., 2011) and have also been implicated to have infra-population regulation mechanisms whereby fecundity per female decreases as the number of worms increases (Bishop and Stear, 2000) which may restrict the ability of FEC to reflect the number of females present in a host.

**Table 2.2** Simulation of the impact of faecal output on the interpretation of faecal egg counts (FEC) based on the feed consumption required to meet the energetic requirement of a 25 kg lamb growing at either 100 or 330 g per day with faecal moisture of either 15% or 30%. Herbage quality is assumed at 11mega joules of metabolizable energy (MJME) per kg Dry matter (DM) with a DM digestibility of 75%. Scenario A: Expected FEC when total egg production is constant at 1,000,000 eggs per day. Scenario B: Expected total egg production when FEC is constant at 500 eggs per g.

| Parameter | Slowing growing lamb | | Fast growing lamb | |
|---|---|---|---|---|
| | Low faecal DM | High faecal DM | Low faecal DM | High faecal DM |
| Live weight (kg) | 25 | | 25 | |
| Liveweight gain (g/d) | 100 | | 330 | |
| Energy requirement (MJME/d) | 7.62 | | 15.1 | |
| Feed intake (g DM/d) | 692 | | 1,376 | |
| Faeces produced (g DM/d) | 173 | | 344 | |
| Faecal DM (%) | 15 | 30 | 15 | 30 |
| Fresh faeces produced (g) | 1154 | 577 | 2,293 | 1,146 |
| Scenario A (Constant egg production) | | | | |
| Total egg production (eggs/d) | 1,000,000 | 1,000,000 | 1,000,000 | 1,000,000 |
| Faecal egg count (eggs/g) | 867 | 1,733 | 436 | 872 |
| Scenario B (Constant faecal egg count) | | | | |
| Total egg production (eggs/d) | 576,934 | 288,467 | 1,146,292 | 573,146 |
| Faecal egg count (eggs/g) | 500 | 500 | 500 | 500 |

Source: (Greer and Sykes, 2012)

### 2.4.2 Pasture larval contamination

Pasture herbage sampling is used primarily for the recovery of third stage larvae on herbage and is expressed as number of $L_3$ larvae per kilogram of pasture dry matter ($L_3$/kg DM) (Familton and McAnulty, 1997).When sampling, appropriate techniques must be taken to avoid error (Couvillion, 1993) as $L_3$ larvae do not migrate far from the faecal pat (Stromberg, 1997) and the distance from which samples are taken from the soil surface can affect the outcome of the results (Crofton, 1948). Further, areas that are immediately around the faecal mass should be avoided during sampling to avoid bias (Taylor, 1939). When faecal aggregation reaches a maximum on pasture there are high refusals of feed intake (Gruner and Sauve, 1982) and refusal of the contaminated area will continue for long periods (Crofton, 1958). Crofton (1954) reported that sampling for *trichostrongyle* parasites can affect the interpretation of results and controlling the variability is more difficult when sampling a whole pasture. They compared the sampling of a whole field to that of some areas in the same field and found larvae recovery of 364 kgDM for whole field compared with 91-182 kgDM in some areas. In addition, Donald (1976) demonstrated that the number of larvae present is dependent on the efficiency of the technique and the quantity of larvae recovered in the sediment. As such, inaccurate sampling of pasture will not give the correct level of infestation (Crofton, 1954) and $L_3$ larvae have spatial pattern of aggregation (Flota-Banuelos *et al*., 2013) on pasture which influences the ability to predict larval challenge as sheep which do not grazed randomly (Crofton, 1954).

### 2.4.3 Herbage Cutting Vs Herbage Plucks

Sampling of pasture herbage when using either the cutting or plucking method can give contrasting results for the recovery of $L_3$ larvae on herbage although some inconsistencies between studies does exist. Verschave *et al*. (2015) found no significant difference of $L_3$ larvae between cutting vs plucking, with 325 ± 479 larvae for every 10 plucks and 305 ± 444 $L_3$/Kg DM for random collection of herbage within four 0.16m $^2$ plots. In addition, Martin *et al.* (1990) found 1890 and 1909 $L_3$/kg DM for 2 measurements totalling 20 cuts in the same area and 1687 and 1878 $L_3$/kg DM when using measurement of 4 plucks in an area. Conversely, Litherland, (2008) found 4021 $L_3$ / kg DM for plucks in contrast to 2715 $L_3$ / kg DM for cuts. Further, Moss and Bray (2006) found 7170 $L_3$/Kg DM and 495 $L_3$/kg DM when cut 15mm above ground level for high-large density and low-small density pasture respectively. In comparison, Rocha *et al*. (2014) found 5694 $L_3$/kg DM for low cutting of 5 cm to the ground and 913 $L_3$ /kg DM for high cutting of 30 cm to the ground. Clearly, variation exists in the method of larval recovery, and herbage sampling, with plucks simulating a grazing animal whereby cuts may provide a better indication of total larvae present.

### 2.4.4 The Baermann Apparatus

The Baermann apparatus is used mainly for the recovery of $L_3$ larvae from herbage, soil and faeces to estimate the number of nematodes present (Bairden *et al*., 1995; Taylor, 1939). The volume of sediment varies slightly when estimating the number of larvae. Young and Trajstman (1980) found 50% of $L_3$ larvae recovered from sediment of 7.5 and 12.5 ml volume, Smeal and Hendy (2009) reported 85% recovery in 5ml sediment volume in contrast to 63.6 and 61.8% when the sediment volumes were between 10 and 15 ml, respectively. In large herbage sample of 500 g wet weight, 90% of $L_3$ larvae were recovered in sediment (Smeal and Hendy, 2009). Similarly, Donald (1967) found 90% of $L_3$ larvae recovered from 25 g of wet weight of small samples. This therefore demonstrate the method of herbage sampling, and the volume of the sediment will determine the numbers of larvae present during each extraction. For excessive amount of soil present, recovery can take up to 8-9 days (Taylor, 1939).

## 2.5 Control options for nematode parasites

Typically control of gastrointestinal nematodes relies on chemotherapy/prophylaxis through the use of broad-spectrum anthelmintic to which parasites are rapidly becoming resistant (Jackson *et al*., 2009; Kaplan and Vidyashankar, 2012; Leathwick *et al*., 2012; Taylor *et al*., 2009; Waller, 2006). Therefore, maintaining animal performance requires new novel and sustainable approaches (Kaplan, 2006; Kenyon *et al*., 2009; Leathwick *et al*., 1992; Waller, 2003). During periods of heavy contamination, animals must be removed from pasture (Brunsdon, 1980) and farmers should practice alternative grazing of different species such as sheep and cattle at short intervals to acquire worms that are less pathogenic (Bairden *et al*., 1995; Morley and Donald, 1980; Southcott and Barger, 1975). Despite knowledge of all these mechanisms, understanding nematode epidemiology and biology and the factors contributing to nematode development outside its host will aid in parasite control (Leathwick *et al*., 1992; Waller, 2003). Nematodes may spend a majority of their life outside of it host with most estimates suggesting around 90% of the population in New Zealand on pastures at one time (Familton and McAnulty, 1997). Therefore, targeting the parasite outside of its host may be a plausible option to assist parasite control. Egg hatching has been shown to be sensitive to both pH and the addition of Nitrogen compounds, utilising treatments that are either acidic or N based seems worthy of investigation. Further details of the possibility of either of these approaches to affect parasite development are described below.

### 2.5.0 Effective Microorganism E.M.

Effective microorganism (EM) consists of five types of microorganisms namely, photosynthetic bacteria, lactic acid bacteria, fermenting fungi, actinomycetes and yeast. The introduction of all these microorganisms in the environment resulted in the breakdown of organic matter and methanogens by anaerobic means (Freitag and Meihoefer, 2000; Lokare, 2007) resulting in a solution with a pH of less than 4. EM has been reputed to also maintain the equilibrium and diversity between beneficial and harmful microorganisms and contains a high bio Carbon and Nitrogen content of 2.24 and 1.48 mg /100g (Wei-jiong *et al*., 1996; Wood *et al*., 1997). Effective microorganism (EM) has been reported to reduce pickle worm by 91% (Higa and Wididana, 1991). In drinking water, it has been reported to increase lamb growth rate by 319 g per day, in comparison to control 286 g per day when applied at the ratio of 1:1000 and on pasture at 10 L per hectare (Chamberlain *et al*., 1997) also indicating it has a very low toxicity to sheep.

### 2.5.1 Effective Microorganism in waste water treatment facility

The use of EM in waste water sewage system is to reduce pathogen, toxins, biodegradable organic materials and heavy metals for environmental safety (Szymanski and Patterson, 2003). Studies on wastewater treatment facilities have shown EM treatment reduced biochemical oxygen demand (BOD) from 2.8 - 0.9, chemical oxygen demand (COD) from 164-109 mg / l and dissolved solid from 2160mg /l to 901 mg/l at pH 7.1 (Namsivayam *et al*., 2011). Similarly, Szymanski and Patterson (2003) reported a significant decrease in pH to 3.1 when the EM dose and the BOD was increased.

### 2.5.2 Effect of Effective Microorganism on egg hatching

Research data of EM on gastrointestinal nematodes is limited. However, previous investigations (Lewis, 2013) have shown that EM can significantly reduce GIN egg hatching by over 95%. These effects were maintained following autoclaving of the EM solution and the effect was consistent with the same effect observed in lactic acid standards at the same pH, which indicates this may be a pH effect rather than an action specific to EM and which appears to directly inhibit larval development inside the egg.

### 2.6 Nitrogen based Fertilizer

### 2.6.1 Effects of Nitrogen based Fertilizers on *Ascaris suum* in waste water treatment facility

Although specific examples of the toxicity of N based fertilisers on ruminant parasites is limiting, there are some examples from alternative species that support the notion that N-based fertilisers may have some toxic effects. Fidjeland *et al*. (2016) showed that ammonia sanitization can inactivate not only bacteria, viruses, protozoans but also helminth eggs. Treatment of toilet waste through the addition of 1-2% urea reduced the recovery of *A. suum* eggs from 1629 to 0 resulting in 100% inactivation after 30 days. Pecson *et al*. (2007) observed a significant decrease in the time for 99% inactivation ($t_{99}$) of *Ascaris* eggs at every pH and ammonia concentration. They then concluded that activation of *Ascaris*

egg is sensitive to ammonia and pH which work with temperature and the duration of time (Pecson and Nelson, 2005). Similarly, Reimers *et al*. (1986) presented ~60% inactivation of *Ascaris* after 10 days in high pH sludge with ~2000 mg/l $NH_3$ added while Kato *et al*. (2001) reported an inactivation of >99% in *Ascaris* egg after 48 h at 37 $^{o}$C and pH 13 in sludge amended with 2600 mg/l $NH_3$. Of the few studies that have investigated the effect of N fertilisers on nematode survival all four N based fertilisers, urea, ammonium nitrate, ammonium nitrate + urea and liquid nitrogen fertilizer were capable of inducing mortality in *H. contortus* $L_3$ larvae, an effect which was dose dependent with the greatest increase in the non-motile $L_3$ occurring at 18 g , see (Figure 2.2) (Howell *et al*., 1999).

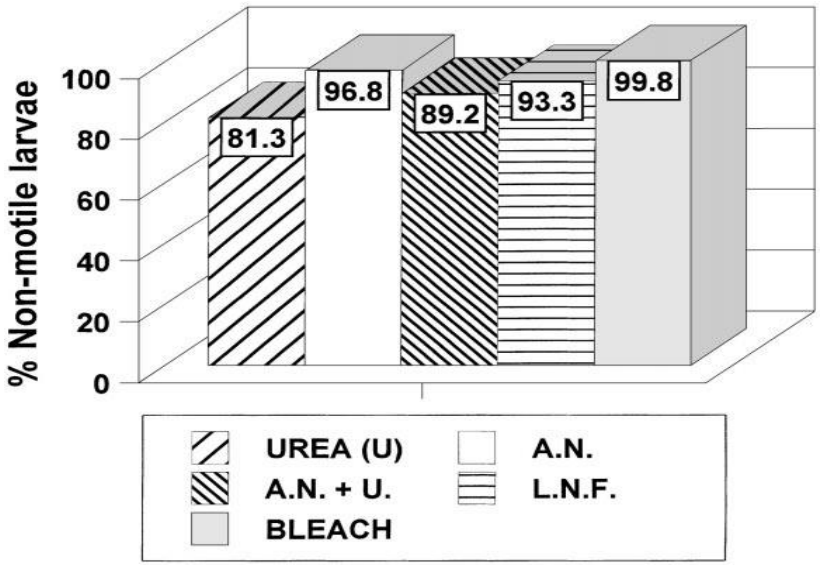


**Figure 2.2** Percent non-motile *H. contortus* $L_3$ larvae when exposed for 4 h to N (18g/100ml) and bleach (10%) solutions. Bars represents least significant difference. Bleach vs. all other compounds; LNF (liquid N fertilizer) vs. all other N sources; Urea (U) vs. ammonium nitrate (AN); AN+ U mixture vs. AN and U ($p< 0.7871$); SE= 0.44.

Recent *in vitro* studies have shown liquid urea at a rate of greater than 6% in solution prevents greater than 95% of *T. colubriformis* eggs from hatching with almost complete inhibition of hatching at a concentration of 20% (Cairns *et al*., 2017). Further, when liquid urea was applied topically to fresh faeces at a rate of 40 units' N per ha, the number of $L_3$ collected following bearmanisation was reduced by 99%, an effect that decreased to 50% when urea was applied to faeces five days old with no effect apparent when applied to eight-day-old faeces (AW Greer, Unpublished data). Overall, these results suggest the effect is specific to egg hatching rather than the larval stages as these authors reported a large number of embryonated, but unhatched eggs still present during the egg hatch assays, an effect which was not able to be reversed following washing provided contact time was more than 6 h (Bennett, 2017, unpublished honours dissertation, Lincoln University).

**2.7 Summary**

Alternatives to anthelmintic are needed to assist parasite control. *In vitro* laboratory studies have indicated that development and hatching of nematode parasite eggs may be sensitive to both low pH and N fertiliser compounds and this may be acting through different mechanisms. However, validation of such an approach in the field is still required. EM is a commercially available product with a low pH of 3-3.5. Conversely, liquid urea is a commercially available product that contains N compounds with a pH of 8.5-9. Validation through field trials that the application of either of these to fresh faeces to reduce nematode egg hatching provides a novel method through which parasite control could be aided by targeting the nematode outside of its host.

# Chapter 3 **Methodology**

### 3.1 Experimental site

The field study was carried out at the Lincoln Sheep Research Farm located in Lincoln, Canterbury, New Zealand from February 9th, 2016 to April 22, 2016. Authorization for the trial was approved by the Lincoln University Animal Ethics Committee, LUAEC#653. In December 2015, one 0.71 ha irrigated paddock of newly sown ryegrass (*Lolium perenne*) and white clover (*Trifolium repens*) mix was established for the start of the trial in February 2016. This area had been cropped in the previous year and no animals grazed the area prior to the start of the study to ensure minimal background larval contamination.

### 3.2 Experimental design

The field study was established as a randomised block design. At day 23 the field was split with temporary electric fencing into eight areas consisting of two blocks measuring 60 m x 40 m that were then each further split into four areas measuring 60 m x 10 m (Figure 3.1). Five-month-old Romney lambs that were naturally infected with gastrointestinal parasites through grazing contaminated pastures and which had not received anthelmintic treatment for a minimum of six weeks were then allowed to rotationally graze the areas to seed the pasture with fresh parasite contamination. Infection with gastro-intestinal nematodes was confirmed prior to the start of grazing using faecal egg count. Lambs were mob stocked to graze each 60 m x 10 m area for sixteen days ie., two days per each of the eight areas. Initially 30 lambs were used, this number was then increased to 45 after four days to ensure low and consistent grazing residuals within each area. To assist with ensuring adequate parasite populations on the pasture for measurement, an additional 6 kg of fresh faeces was collected from ten-month-old Hampshire lambs that were housed and monospecifically infected with either *Trichostrongylus colubriformis* or *Teladorsagia*, with a concentration of 800 eggs per g (epg) was evenly spread onto each area post-grazing from day 12. Following the first eight days in block one, the area was irrigated (K-line sprinkler) at the application rate of 20 mm for 12 hours to assist parasite larval development and the procedure continued after the second eight days shift in block 2 at the same application rate.

Immediately following each shift, i.e., every two days, the 60 m x 10 m grazed block was split again into six 10 m x 10 m plots, demarked by lines mown to ground level with a rotary hand mower. Within each block, plots were randomly allocated to one of three treatments, each replicated twice. The treatments were: topical application of urea (Flowfert N, Ravensdown Ltd) at the equivalent of 200 L or (40 units of N) per ha, topical application of EM mixture (Nature farm Ltd) at 200 L per ha or water sprayed at 200 L per ha. Treatments were applied using a hand sprayer and were only applied after the initial grazing by the lambs providing the seed contamination.

The order of treatments remained the same within each block so that the adjacent plots in the grazing rotation received the same treatment. The order of treatments was then re-randomised for the second block of four grazing areas. After the first sixteen (16) days of complete rotation, the animals were removed from the trial area.

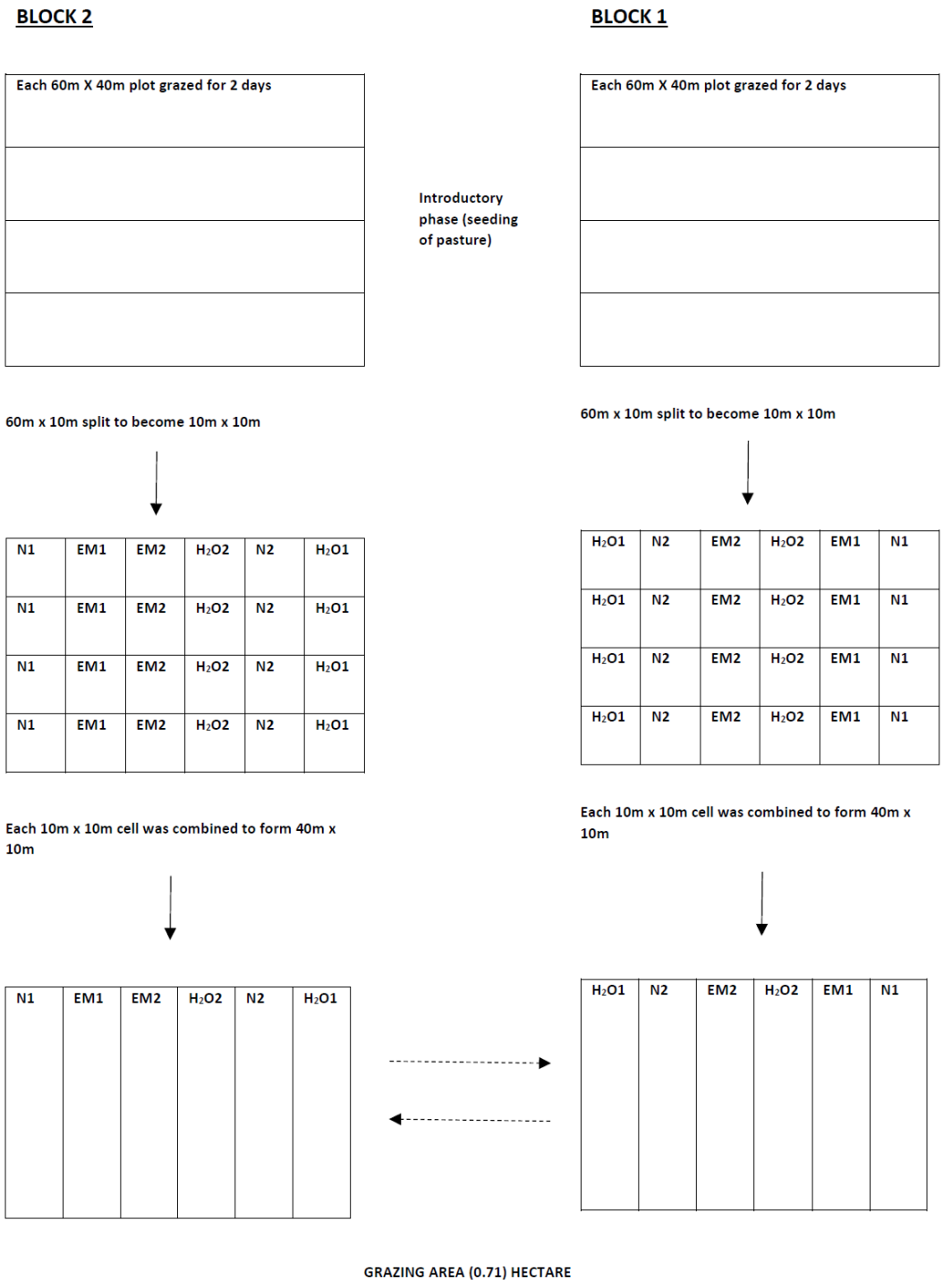


**Figure 3.1 Schematic diagram of the treatment plots**

### 3.3 Grazing Study

The effect of treatment on lamb performance and parasitological parameters was evaluated in a grazing trial. At the completion of the initial seeding grazing rotation (day 17), 30 (5-month-old) Romney lambs were selected from the group of 45 and were weighed and treated with anthelmintic to remove resident parasite burdens (Trio sheep drench, Ravensdown Animal Health, Christchurch) which was confirmed with the faecal egg count. Following drenching, lambs were grazed in the remainder of the paddock unused in the initial rotation for the following 7 days in two shifts to ensure the effects of the anthelmintic treatment had worn off and were not likely to influence larval establishment.

At day zero, the second phase of the field trial began. Lambs were then re-weighed and allocated hierarchically by live weight to one of six groups (n=5) then each group was randomly allocated to graze each of the treatment plots. Animals were introduced into the first of their respective 10 m x 10 m plots, divided by temporary electric fencing on day 0, starting with the area that was grazed first during the seeding rotation. For block one, animals within the same treatment and replicate groups graze each area for two days before being moved to the adjacent area and the procedure continued until they had completed eight days grazing, or four movements, in block one. After the first four shifts, animals were moved to their respective treatments in the second block with the same protocol followed. Following the initial 16-day rotation, sampling of the animals continued as far as possible past the 21-day pre-patent period of most gastro-intestinal nematodes.

Animals were only removed from their plots for faecal sampling and weighing (detailed below) and were not allowed to graze any other treatment area. In rotation two, the four 10 m x 10 m plots were combined to form 40 m x 10 m starting from block one and the same procedure continued in block two. Lambs grazed each of the six plots that received the same treatment for one week within each block. Six lambs, one from each group, were removed on day 24 to reduce grazing pressure and placed into a clean parasite free paddock based on pasture larval sampling. On day 32 the remaining twenty-four lambs joined the other six due to insufficient pasture regrowth in the treatment plots. Faecal sampling continued for a further 17 days to extend beyond the pre-patent period of any contamination to be picked up from the treatment areas but remain within the pre-patent period of the adjacent paddock to minimize the chances of detecting any contamination post-shifting of the plots.

### 3.4 Measurements and sampling

The effect of treatment on nematode larval development was assessed utilising two primary methods, *viz*, pasture larval concentration and through the grazing of lambs, each of which are described below.

### 3.4.1 Pasture Larvae

Herbage samples were collected for the determination of the concentration of nematode larvae on pasture taken immediately prior to each grazing. Initial measurements for each plot were recorded prior to seeding and again at 23 days after treatment was applied, giving eight replicates of treatment across time and two replicates at each time, sixteen replicates in total. To determine the total number of larvae present, grass cutting scissors was used to cut grass at ground level with samples taken every two steps in an X pattern in each of the respective plots at 9 am each morning. Herbage samples were placed into plastic bags at the time of collection and stored at 4°C until processing. All samples were processed within a week of collection.

During processing, the plastic bags with samples were weighed and 4 litres of lukewarm water was added. The plastic bag was then tied and put in a small hand washing machine with 200 revolutions per minute for 3 minutes. After washing a small incision was made in the bag through which the fluid was drained onto a coarse mesh sieve (aperture size 2 mm) into a beaker. The herbage that remained in the bag was removed and rinsed gently. The collected suspension was left to settle overnight at 4°C with the fluid siphoned off leaving sediment and larvae which were transferred to a measuring cylinder for a second sedimentation. After 36 hours, 100 ml of fluid was withdrawn and stored in glass bottles. After storage at 4°C the sample was reduced in volume to 20 ml by siphoning and the larvae present in two x 1 ml sample were counted and their species differentiated. For the herbage samples, after squeezing, it was spread on a tray then dried in an oven at 70°C. The dry herbage was weighed, and the fresh grass weight was used in the final estimation of numbers of larvae per kilogram of fresh herbage was calculated. The method of bearmann apparatus measurements of larvae per herbage were calculated as (Number of larvae /kg fresh herbage = Number of larvae counted x (ml sediment / ml sed-ml subsample) x (1000/grass wt). The total larvae per plot was calculated using the dry matter percentage x the post grazing pasture mass x the grazing area and the number of larvae per count. The cumulative larval challenge was determined by the pre grazing herbage mass x $L_3$/kgDM.

### 3.4.2 Animal measurement

Lamb live weight and faecal samples were recorded every 8 days from day 0 until day 48. For live weight, animals were fitted with an electronic tag and weights were recorded for each individual utilising a Prattley auto drafter (Prattley Industries Ltd, Temuka, New Zealand) fitted with Tru-Test load bars and a Tru-test XR3000 head unit (Tru-test Ltd, Auckland, New Zealand) and an Aleis electronic tag reader (Aleis Ltd, New Zealand) with a sensitivity of 0.2 kg. All live weights were recorded immediately

following removal from pasture at 9 am with fasted live weights following 8 h fasting recorded on day 48.

**3.4.3 Faecal egg count**

For faecal egg count, faecal samples were taken directly from the rectum of each lamb and were stored at 4°C until processing which occurred within 24 hrs. The method of analysis was the Modified McMaster technique (M.A.F.F, 1977). Briefly, a total of 1.7 g of faeces from each sample was weighed and placed into a jar containing 10 ml of water and left to soak overnight at 4°C to soften. The following day, 40 ml of saturated sodium chloride (NaCL) solution was added and the sample homogenised for 25 seconds with an electrical stirrer. A Pasteur pipette was used to fill both chambers of a moistened McMaster slide with the faecal suspension. The number of eggs present in both chambers of the slide were counted under a microscope, totalled and multiplied by 100 to give the number of eggs per gram (epg) of fresh faeces with a sensitivity of one hundred eggs per gram. Total estimated daily faecal egg output was determined by the percentage organic matter digestibility OMD x number of animals per proup dry matter intake DMI prior to faecal sampling /0.15*1000*FEC/100(epg).

**3.4.4 Pasture Measurements**

Herbage mass was measured pre-and post-grazing with a rising plate meter to determine the herbage mass (kgDM) in each area. Measurements were taken every two (2) steps in an X pattern within each plot. The rising plate meter was calibrated to the pasture sward using 10 quadrat cuts taken at various pasture heights. Briefly, 10 recording of herbage mass within each quadrat were recorded with the plate meter with the mean number of 'clicks' recorded. Herbage was cut to ground level using grass cutting scissors, the samples then weighed after being placed in an oven at 60°C for 24 hours. Dry weight of the herbage from each quadrat was recorded, converted to kgDM per ha and regressed against the mean number of clicks to obtain an equation for the number of clicks to describe the kgDM per ha with the equation: *herbage mass (kgDM per ha)* = 37.223 *x* # clicks + 27.689 where *x* = mean number of clicks from the rising plate meter. The cumulative larval challenge was calculated from the concentration of larvae on herbage (larvae per kgDM) x the amount of herbage in each plot. The plot size was 10 m x 10 m or (100 $m^2$) and 40 m x 10 m or (400 $m^2$). The rising plate meter gives the amount of dry matter per ha.

**3.4.5 Metabolisable Energy Analysis (ME)**

Herbage samples were collected from 12 areas of 40 m x 10 m plots viz two blocks x 6 treatments, then freeze dried. The dried samples were ground and passed through a 1 mm sieve and were analysed by a near infrared spectrophotometer (NIRS Model: FOSS NIRS Systems 5000, Maryland USA). The crude protein (CP), organic matter digestibility (OMD), organic matter percentage OM% and dry matter digestibility (DMD) were used to estimate the metabolizable energy from the equation: ME = average OM % / 100* average OMD/100. The ME range between 12.2 -12.6 (MJ/Kg DM) and the ME

(MJME/KgDM) = DOMD x 0.16 for each treatment. The method of analysis was adopted from (Alderman *et al*., 1993).

### 3.5 *In vitro* larval culture

The effect of treatments under controlled conditions was deterimed using *in vitro* larval culture. Sheep faeces was collected from 10 month old Hampshire lamb using an improvised harness fitted over the peri-anal area of the sheep. Faeces were less than 24 h old when applied to the pasture on day 12 where a 600 g subsample was divided into six 100 g samples and evenly spread across six separate plastic trays. Each tray was placed onto the ground on one plot prior to the treatment application and subjected to the same application as the reminaing ground surface in the plot. The trays were left overnight on each of the treatment plots before being taken to the laboratory. In the laboratory each tray was covered with a plastic bag with small holes punched in it for areation and was then placed in a climate room at 25°C for 10 days to allow larvae to develop.

At day 10 of culture, the faeces from each tray was placed into individual paper towel with an elastic band to provide support from spillage. Individual treatments were then placed into a glass bearmann funnel with 2 L of luke warm water for a period of 48 hours to allow larvae to migrate through the tissue and collect at the bar of the funnel. After bermanisation 100 ml fluid was drawn from the base of the funnel into a glass bottle and left to settle overnight. From the 100 ml fluid, 50 ml was siphoned off and an additional 50 ml was added from the bearman funnels to equivalent 100 ml fluid. This was then refrigerated for four hours at 4°C. The refrigerated suspension from the glass bottle was reduced to volume of 20 ml by siphoning. Larvae present in five 200 microliter (1 ml total) samples were counted and multiplied by the volume to give the total number of larvae collected.

### 3.6 Second *in vitro* larvae culture 'with and without plastic bag method'

Faeces were collected from additional male sheep that were housed indoors and with a FEC averaging 800 eggs per gram (epg) faeces. A total of 200 g and 160 g of faeces were taken one week apart. The method of 'with plastic bags' and 'without plastic bags' was used to determine which method work best in the control environment. Infected sheep faeces was homogenized then divided into four equal quarters of 50 and 40 g. Faeces were placed in plastic trays with treatment liquid urea solution and water, the 'with plastic bags' and 'without plastic bags' method was applied for each treatment and replicate. A total of 1.7 g urea fertilizer was dissolved into 4 ml of hot water, which was then divided into 2 ml for each treatment and replicate. For the water treatment 4 ml of water was divided into 2 ml for each treatment and replicate. Faeces were sprayed topically with liquid urea solution and water and was replicated twice with each treatment receiving the same 2 ml before cultured at 25°C for 10 days. Following culture, the same procedure of bermanisation was applied as in the first *in vitro* trial.

For the ‘with plastic bags’ method, plastic bag with small holes for aeration were used to cover the plastic containers, while for the‘without plastic bags’ method, no covering were placed on the plastic containers in the control room and was subjected to the same climatic condition as the plastic bag method.

**3.7 Statistical analysis**

All statistical data were analysed using GENSTAT (Release 16, VSN international Ltd) suite of statistical package. For pasture larvae sample, data was subjected to general analysis of variance (one-way ANOVA) to test for differences of means of each variable with each time point and replicates within each point as the replicate of treatment, giving sixteen replicates in total. Repeated measures were used to analyse changes in live weight, faecal egg count, herbage mass, dry matter intake, cumulative larval challenge and faecal egg production. This was to determine the effect of time and treatment and the difference between the means of each treatments at each time of measurements. Faecal egg count and pasture larvae samples were log transformed where log 10 (x+1) to stabilize the variances in one-way ANOVA and Turkey test was used to compare the significant difference between each treatment. For larval culture, the number of larvae recovered was analysed using ANOVA with differences determined post hoc with a Turkey test.

# Chapter 4 Results for field study

## 4.1 Field Trial

### 4.1.1 Pasture Mass

Pasture herbage mass pre-and post-grazing for each rotation are given in Figures 4.1-4.3. Overall, there was no effect of treatment or time (P>0.05 for all). Pasture quality was not different between treatments (P>0.05 for all), with total organic matter digestibility (OMD) being 87% for all, the MJME/kgDM being 12.3,12.4 and 12.4 and crude protein being 32.0, 31.6 and 31.2 for water ($H_2O$), 20% liquid urea solution and Effective microorganism (EM), respectively.

Mean pre-grazing and post- grazing herbage mass for water ($H_2O$), treatment is presented in (Figure 4.1). Pre-grazing pasture herbage mass in rotation 1 was 4164 ± 614 KgDM which then declined to 2073 ± 62 KgDM in rotation 2 with respective post grazing herbage mass being 870 ± 145 and 516 ± 2 KgDM / ha.

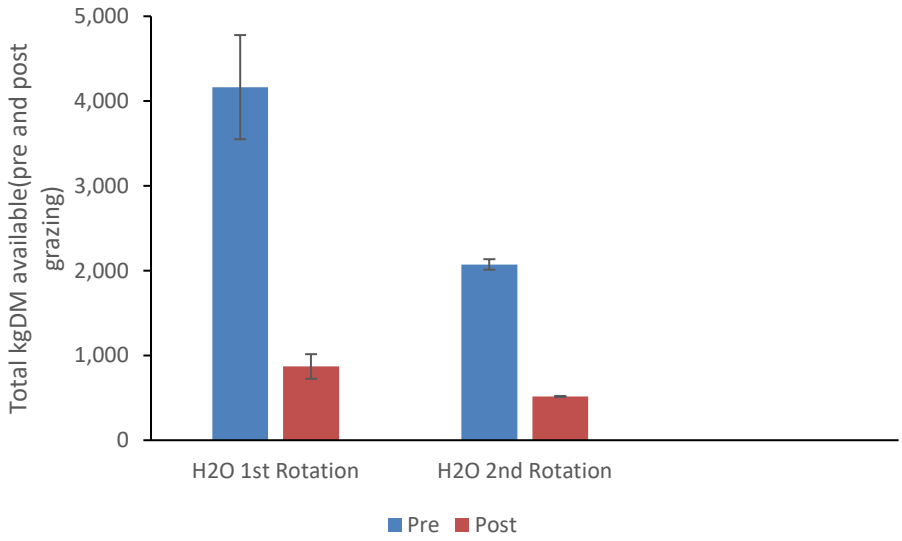


**Figure 4.1. Mean Pre-and Post-Grazing Herbage mass (kgDM available) for plots treated with water ($H_2O$), 16 and 23 days post treatment. Plots areas were 100m$^2$ during first grazing rotation and 400m$^2$ during second grazing rotation.**

Mean pre-grazing and post grazing herbage mass for 20% liquid urea solution (N) treatment is presented in Figure 4.2. Pre-grazing pasture herbage mass in rotation 1 was 3575 ± 575 kgDM which then declined to 1934 ± 176 kgDM in rotation 2 with respective post grazing herbage mass of 720 ± 117 and 533 ± 33 kgDM per ha.

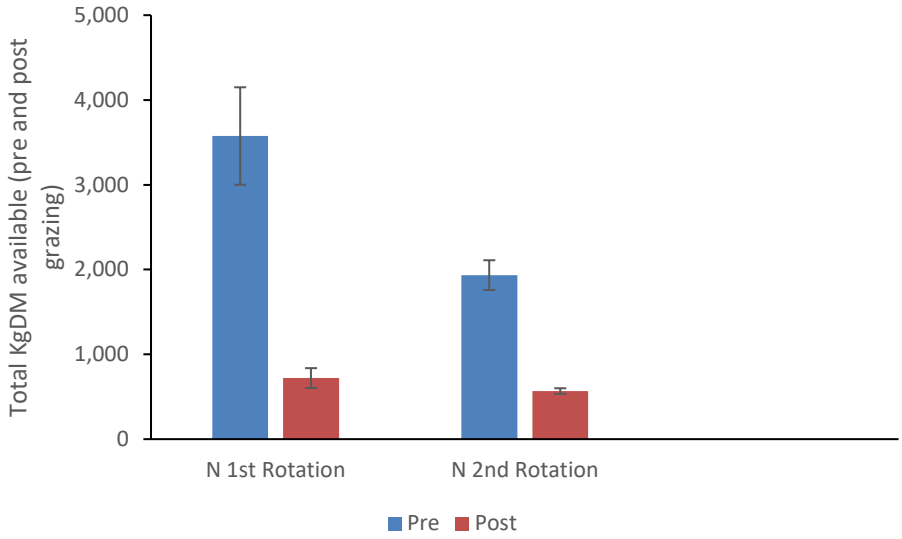


**Figure 4.2. Mean Pre-and Post-Grazing Herbage mass (KgDM per ha) for plots treated with 20% liquid urea solution (N) for 16 and 23 days post treatment. Plots size were 100m$^2$ during first grazing rotation and 400m$^2$ during second.**

Mean pre-grazing and post grazing herbage mass for EM is presented in Figure 4.3. Pre-grazing pasture herbage mass in rotation 1 was 4188 ± 792 kgDM and 2235 ± 366 kgDM in rotation 2 with respective post-grazing herbage mass of 788 ± 123 and 537 ± 35 kgDM per ha.

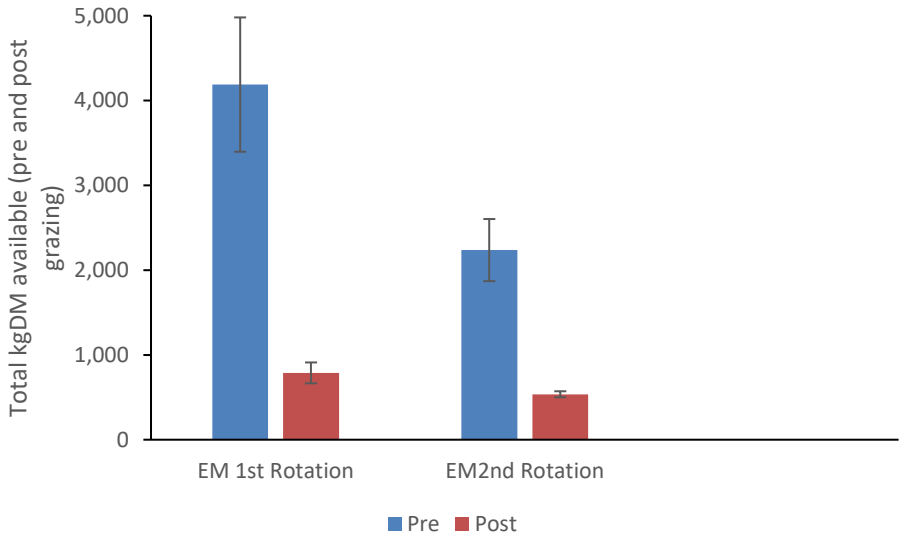


**Figure 4.3. Mean Pre-and Post-Grazing Herbage mass (KgDM per ha) for plots treated with EM for 16 and 23 days post treatment. Plots size were 100m$^2$ during first grazing rotation and 400m$^2$ during second.**

### 4.1.2 Pasture Larvae Contamination

The number of both *Nematodirus* and S*trongyl*e ($L_3$ per kgDM) larvae present at the start of each grazing are given in Figures 4.4 and 4.5**,** respectively. High pasture larval numbers were observed on two occasions, being one EM replicate on day 10 and one N replicate on day 17, *viz,* 116,393 and 49,296 $L_3$ larvae per kgDM, for *Strongyle* and *Nematodirus,* respectively. These were considered outliers and were removed from the preliminary analysis as they were only shown in one replicate. Data with and without days 10 and 17 included is also presented.

#### 4.1.2.1 Strongyle $L_3$ per KgDM

The total number of *Strongyle* $L_3$ larvae present on pasture is presented in Figure 4.4. Overall, pasture $L_3$ *Strongyle* larvae contamination was not influenced by treatment (P=0.05), nor was there a treatment x time interaction (P=0.05). For the water ($H_2O$) in rotation 1, 1266 $L_3$ per kgDM were found, which then decline to 136 $L_3$ in rotation 2 and increased to 559 $L_3$ per kgDM in rotation 3. For EM treatment, pasture *Strongyle* $L_3$ larvae were 2295 $L_3$ per kgDM in rotation 1, then decline to 695 $L_3$ per kgDM in rotation 2 and increased to 3838 $L_3$ per kgDM in rotation 3. For the 20% liquid urea solution (N) treatment, 1087 *Strongyle* $L_3$ per kgDM were present in rotation 1 with no larvae found thereafter in rotations 2-3.

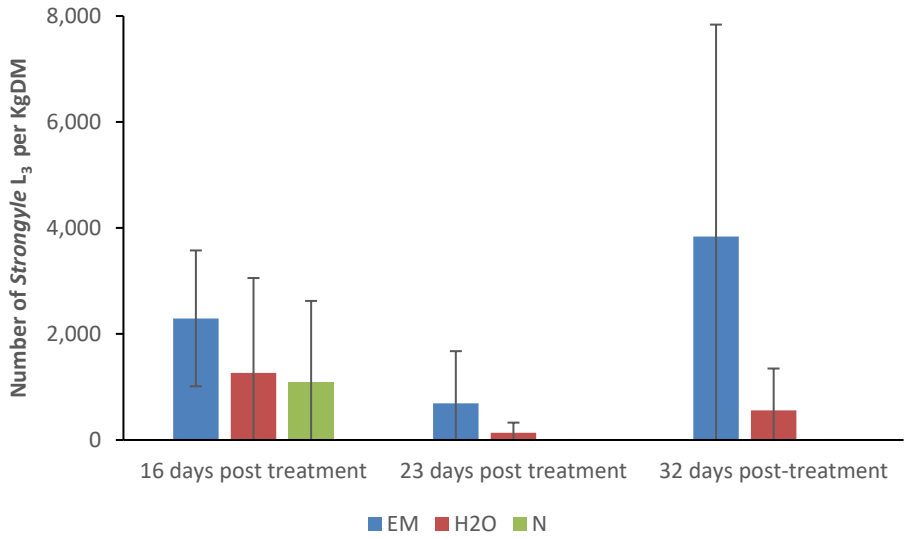


**Figure 4.4. Number of *Strongyle* $L_3$ per KgDM recovered from plots that were treated with either water ($H_2O$), 20% liquid urea solution (N) or effective micro-organism mixture (EM) 16 days, 23 and 32 days post treatment.**

### 4.1.2.2 *Nematodirus* Larvae per kg DM

The number of *Nematodirus* $L_3$ recovered per kgDM are given in Figure 4.5. Overall, there was an effect of time (P=0.05) but not treatment (P>0.05) or treatment x time interaction (P>0.05). For water ($H_2O$) treatment a mean of 2022 $L_3$ larvae per kgDM were present during rotation 1, which then declined to 368 $L_3$ in rotation 2 and zero on day 32. For EM, 1739 $L_3$ per kgDM were found in rotation 1 which then decreased to 1116 $L_3$ during rotation 2 and zero on day 32. While for 20% liquid urea solution (N) treatment, 630 $L_3$ per kgDM were found in rotation 1 which then declined to 0 in rotation 2 and day 32.

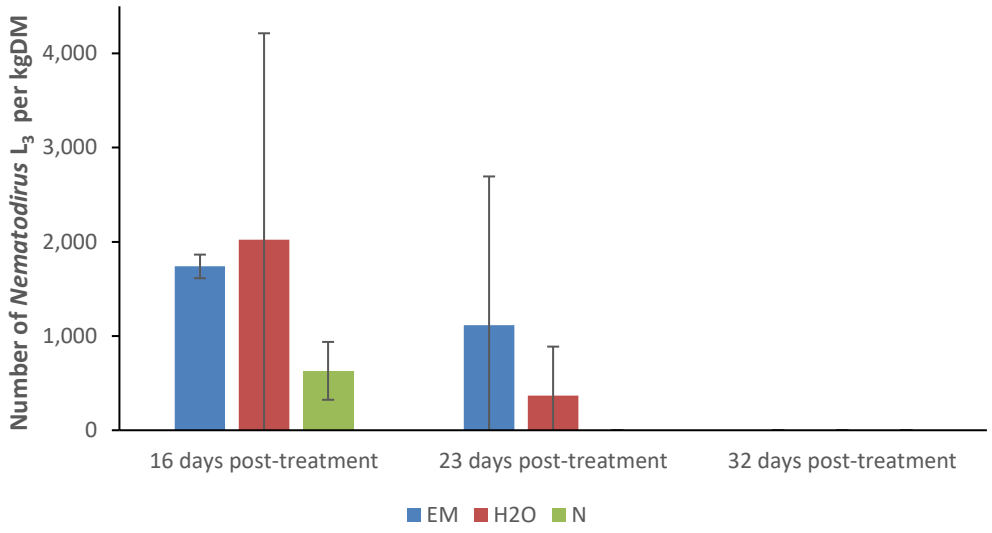


**Figure 4.5. Number of *Nematodirus* $L_3$ per kgDM in plots that were treated with either water ($H_2O$), 20% liquid urea solution (N) or effective micro-organism mixture (EM) 16 days post treatment after the first grazing rotation and 23 days post treatment after the second grazing rotation.**

### 4.1.2.3 Cumulative apparent *strongyle* intake per treatment plots with outlier days included and removed

Arithmetic mean cumulative apparent *Strongyle* intake per plots with outlier data from days 10 and 17 included is given in Figure 4.6. There were greater increases of 375681, 67677 and 39038 $L_3$ per kgDM observed in the EM, N and $H_2O$ treated plots respectively. There was no effect of treatment (P>0.05) or treatment x time interaction (P>0.05) reflecting increases in one replicate only.

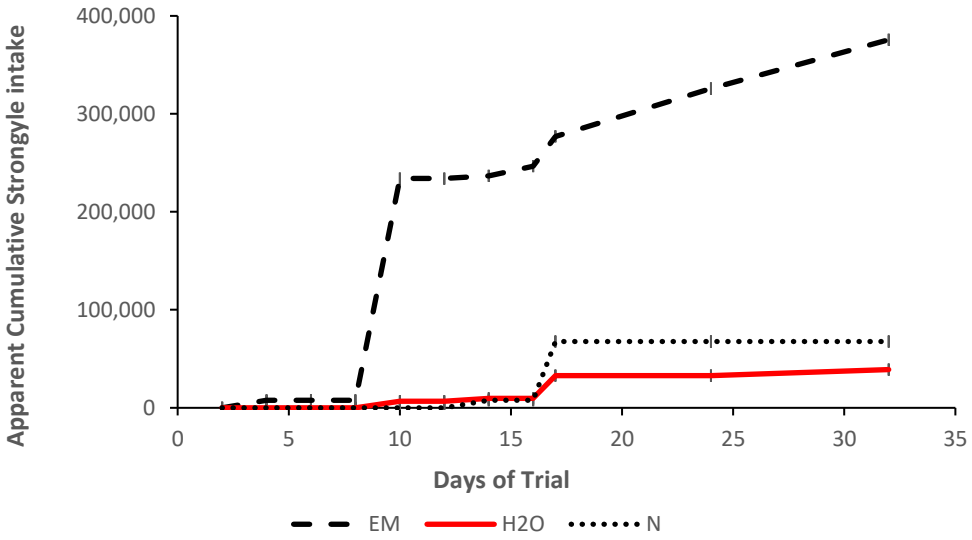


**Figure 4.6. Total apparent cumulative *Strongyle* larval intake per plot with data from the outlier on day 10 and 17 included for treatment with either water ($H_2O$), 20% liquid urea solution (N) or effective micro-organism mixture (EM) during the first grazing rotation after treatment (D1-16) and second grazing rotation (D17-31). Plots size were 100m$^2$ during first rotation and 400m$^2$ during rotation 2.**

Arithmetic mean calculated apparent cummulative *Strongyle* intake with the outlier values from day 10 and 17 removed are given in Figure 4.7. Increases of 118905 $L_3$ per kgDM were observed in the EM treatment compared with 15643 and 7739 $L_3$ per kgDM for water and N respectively. Overall, there was an effect of both time (P=0.003) and treatment (P=0.001) reflecting an increase with time of cumulative apparent larval intake that was greater in the EM treatment than water of urea treatments.

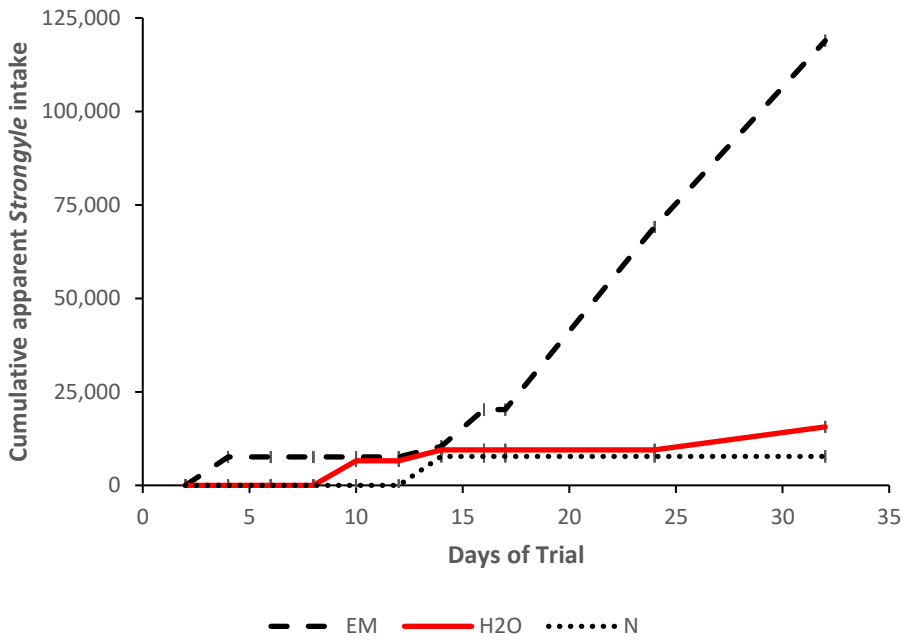


**Figure 4.7. Total apparent cumulative *Strongyle* intake larvae per plot with data from the outliers on day 10 and 17 removed for treatment with either water ($H_2O$), 20% liquid urea solution (N) or effective micro-organism mixture (EM) during the first grazing rotation after treatment (D1-16) and second grazing rotation (D17-31). Plots size were 100$m^2$ during first rotation and 400$m^2$ during rotation 2.**

### 4.1.2.4 Cumulative apparent *Nematodirus* intake per treatment plots with outlier days included and removed

Arithmetic mean cumulative apparent *Nematodirus* intake with data from day 17 included is given in Figure 4.8**.** Overall, there was an effect of time (P=0.05) but there was no effect of treatment or treatment x time interaction (P>0.05 for both) reflecting similar increases in all treatments from day 17.

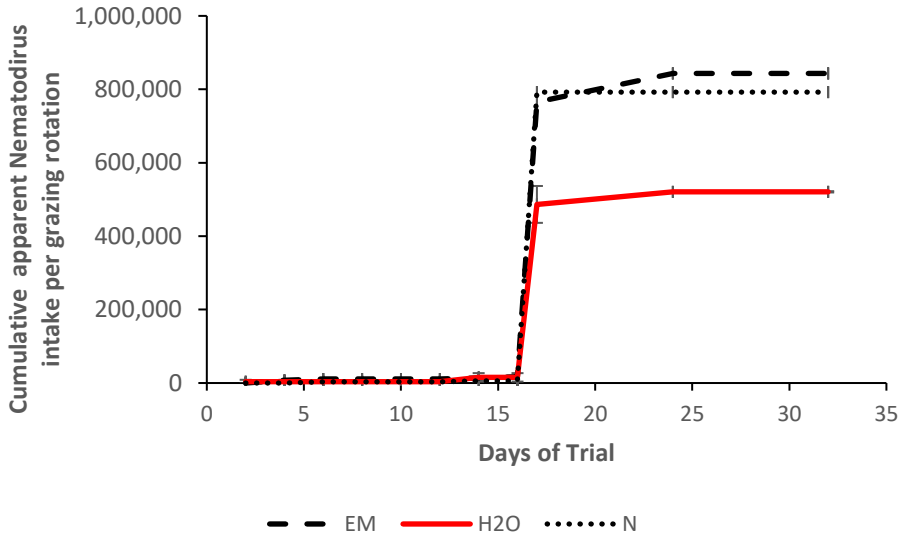


**Figure 4.8. Total apparent cumulative *Nematodirus* $L_3$ intake with data from the outlier on day 17 included for treatment with either water ($H_2O$), 20% liquid urea solution (N) or effective micro-organism mixture (EM) during the first grazing rotation after treatment (D1-16) and second grazing rotation (D17-31). Plots size were 100$m^2$ during first rotation and 400$m^2$ during rotation 2.**

Arithmetic mean apparent cumulative *Nematodirus* larvae intake from each plot with data from day 17 removed from all treatment groups is given in Figure 4.9. Overall, there was an effect on treatment (P=0.004) and time (P=0.007) reflecting a lower cumulative larval challenge in N treatment group although this was not reflected in a treatment x time interaction (P=0.810).

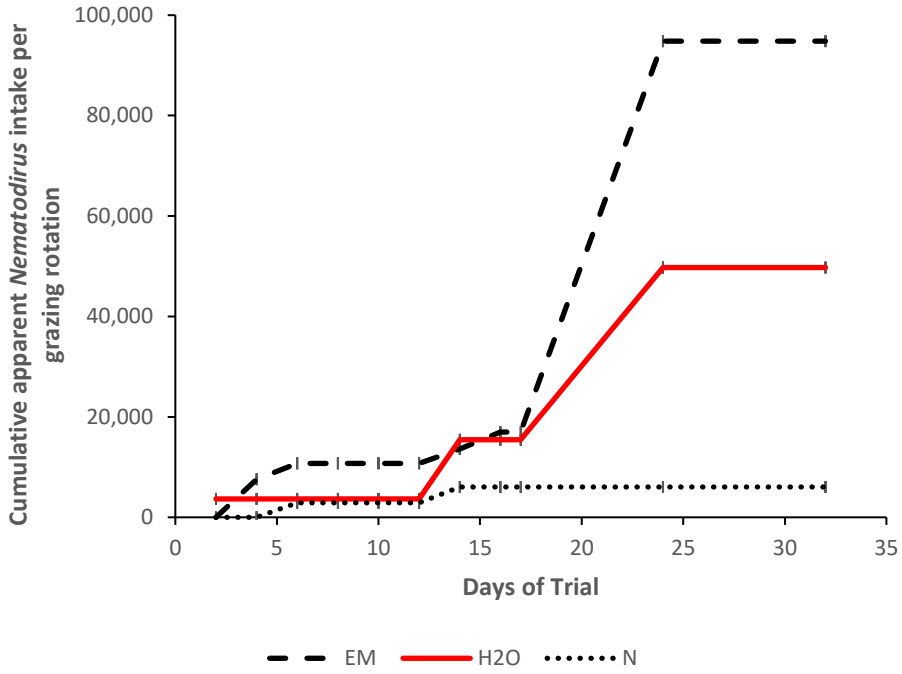


**Figure 4.9. Total apparent cumulative *Nematodirus* $L_3$ larvae intake per plot with data from the outlier on day 17 removed from treatment with either water ($H_2O$), 20% liquid urea solution (N) or effective micro-organism mixture (EM) during the first grazing rotation after treatment (D1-16) and second grazing rotation (D17-31). Plots size were 100m$^2$ during first rotation and 400m$^2$ during rotation 2.**

### 4.1.3 Faecal Egg Count

#### 4.1.3.1 *Strongyle* FEC (epg)

Arithmetic mean stronglye FEC (epg) is given in Figure 4.10. Overall, there was an effect of time (P=0.001) but no effect of treatment (P=0.716) or treatment x interaction (P=0.997) reflecting no eggs present in any treatment group until 17 days after the start of the grazing study folowed by similar increases in FEC (epg) in all three treatment groups to between 560 and 730 epg by day 48.

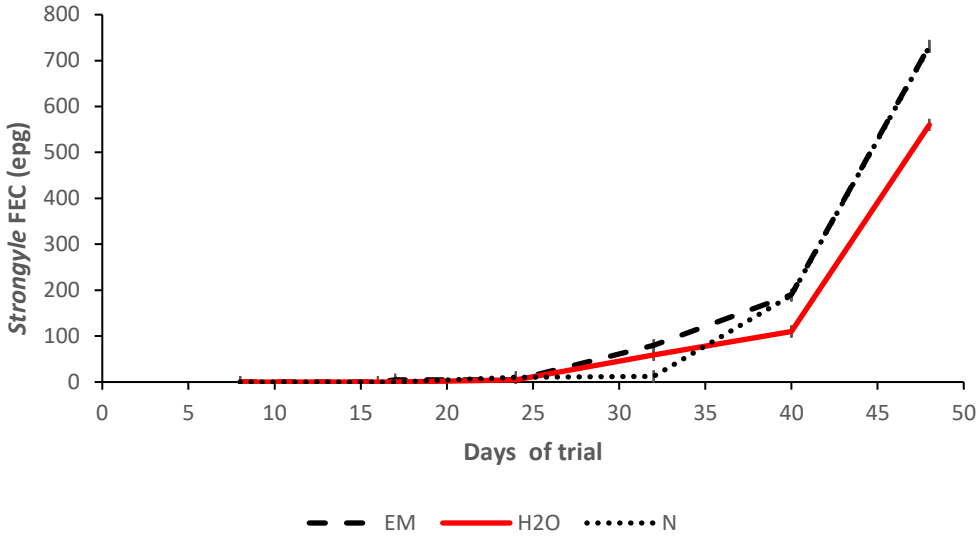


**Figure 4.10. Arithmetic mean *Strongyle* FEC (epg) from day 1-32 for animals grazing areas that had received the equiavalent of 200 l per ha of either water ($H_2O$), effective microorganism mixture (EM) or 20% liquid urea solution (N). Animals were removed from treatment plots at day 32 and followed up to day 48 in a non-treatment paddock.**

### 4.1.3.2 *Nematodirus* FEC (epg)

Arithmetic mean *Nematodirus* FEC (epg) are given in Figure 4.11**.** Overall, there was no effect of treatment (P=0.726) or treatment x time interaction (P=0.748) reflecting low mean values in all treatment groups that did not vary with time or with treatment.

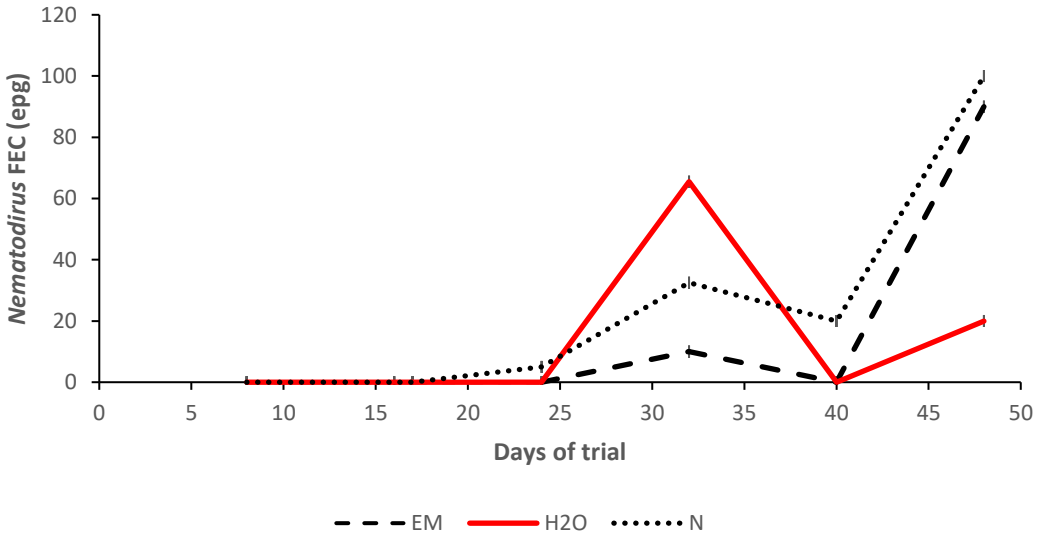


**Figure 4.11. Arithmetic mean *Nematodirus* FEC (epg) for animals grazing areas that had received the equiavalent of 200 l per ha of either water ($H_2O$), effective microorganism mixture (EM) or 20% liquid urea solution (N). Animals were removed from treatment plots at day 32 and followed up to day 48 in a non-treatment paddock.**

### 4.1.3.3. Estimated *Strongyle* faecal egg production

The total estimated daily faecal egg production per animal for *Strongyles* is presented in Figure 4.12**.** Overall, there was an effect of time (P=0.004) but no effect of treatment (P=0.21) or treatment x time interaction (P=0.126), reflecting faecal output increased with time from day 17 to 32.

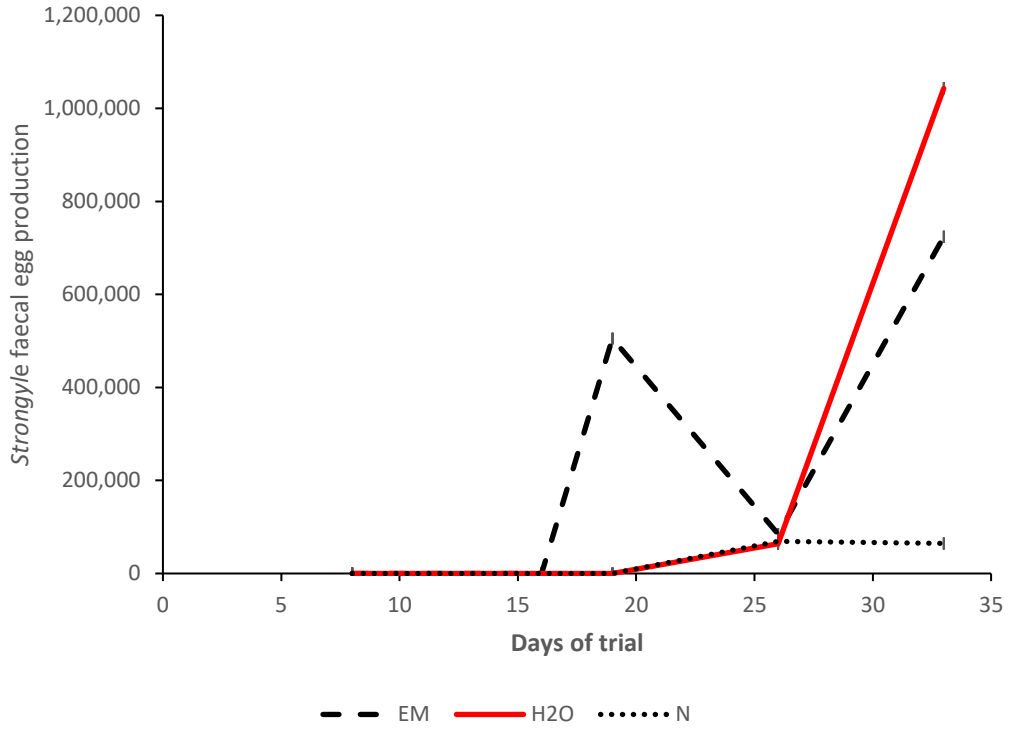


**Figure 4.12. Total estimated daily *Strongyle* faecal egg output for animals grazing areas that had received the equiavalent of 200 l per ha of either water ($H_2O$), effective microorganism mixture (EM) or 20% liquid urea solution (N). NB. Faecal output was only estimation up until day 32 when lamb were grazing plots that were measured for pre and post grazing mass.**

### 4.1.3.4 Estimated *Nematodirus* faecal egg production

The total estimated faecal egg production is presented in Figure 4.13**.** Overall, there was an effect of time (P=0.02) but no effect of treatment (P=0.219) or treatment x time interaction (P=0.175) reflecting increases from day 26 to 32 in all treatments.

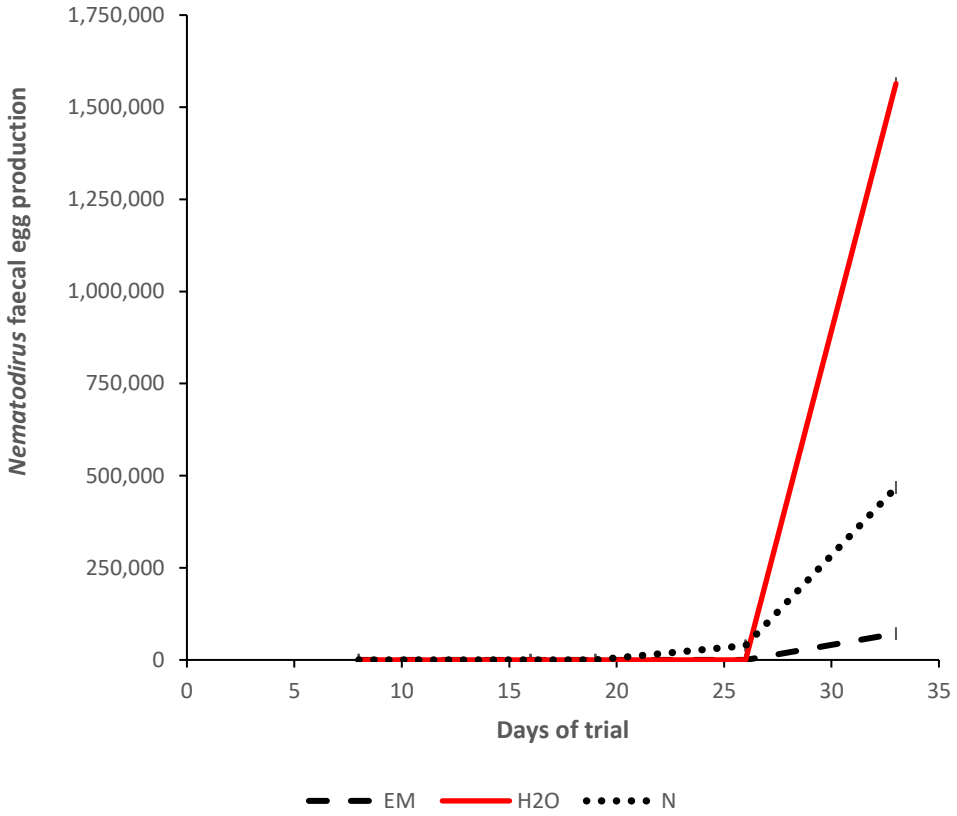


**Figure 4.13. Estimated *Nematodirus* faecal egg production for animals grazing areas that had received the equiavalent of 200 l per ha of either water ($H_2O$), effective microorganism mixture (EM) or 20% liquid urea solution (N). NB. Faecal output was only estimation up until day 32 when lamb were grazing plots that were measured for pre and post grazing mass.**

### 4.1.4 Animal Performance

Mean LW for lambs grazing treatment groups water ($H_2O$), urea (N) and EM is presented in Figure 4.14. Overall, there was an effect of time (P=0.001) reflecting an increase in the LW of all groups with time but there was no effect of treatment (P=0.62) or a treatment x time interaction (P=0.58).

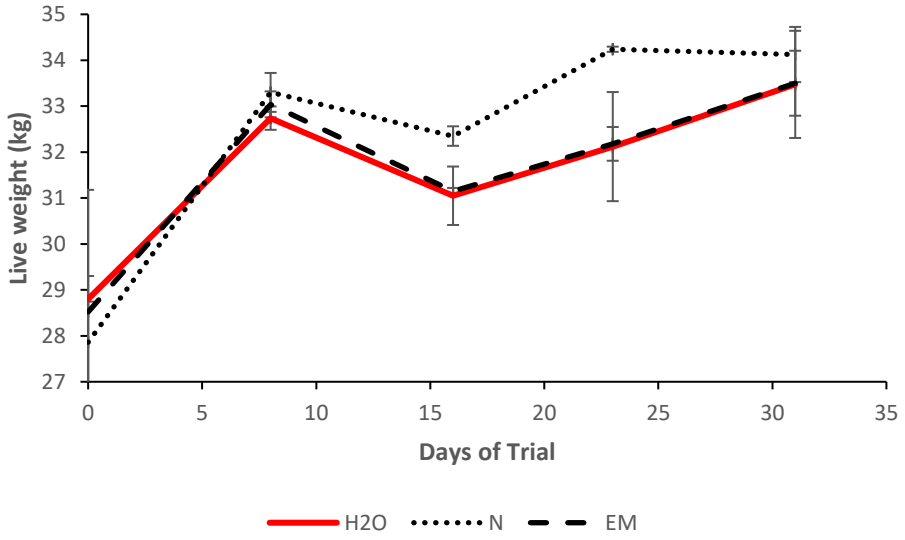


**Figure 4.14. Mean LW (kg ± s.e.m) for lambs grazing pastures treated with water (H2O), 20% liquid urea solution (N) or effective micro-organism mixture (EM) post grazing.**

Mean total live weight (LW), estimated dry matter intake (DMI) by disappearance and feed conversion efficiency (FCE) per plot for the animals grazing each treatment group between days 0 and 32 are given in Table 4.1. Overall, mean LW was not different between treatments (P=0.45) despite the N treatment being 50% greater than the water treatment. Total estimated DMI per plot was not different between treatment groups (P=0.47) and feed conversion efficiency was lowest in the water treatment group and greatest in N treatment group although not statistically significant (P=0.41).

**Table 4.1 Mean live weight (LW; kg per treatment), total dry matter intake (DMI; kgDM) and feed conversion efficiency (FCE; gLW per kgDM consumed) from day 1-32 for animals grazing areas that had received the equiavalent of 200 l per ha of either water ($H_2O$), effective microorganism mixture (EM) or 20% liquid urea solution (N).**

| Variable | EM | N | Water | P value |
|---|---|---|---|---|
| Mean live weight (kg) | 24.30 ± 12.30 | 33.50 ± 6.93 | 22.20 ± 2.55 | P=0.45 |
| Dry matter intake (kgDM) | 131.31 ± 6.72 | 113.1 ± 21.4 | 124.57 ± 4.40 | P=0.47 |
| Efficiency (gLWG/kgDM) | 187.7 ± 103.3 | 307.5 ± 119.5 | 178.7 ± 26.7 | P=0.41 |

# Chapter 5 Results for *in vitro* studies

## 5.1 *In vitro* larvae culture when topically applied

Mean total number of larvae recovered per g faeces are given in Figure 5.1. Compared with the water treatment, larval recovery was reduced by 98% in the N treatment (P=0.001) and increased by 2.5-fold in the EM treatment (P=0.31).

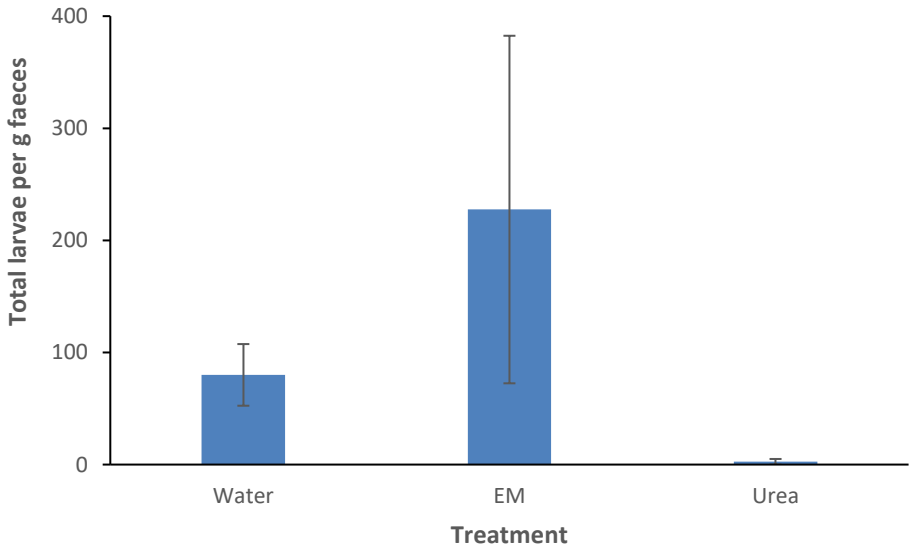


**Figure 5.1. Number of $L_3$ larvae recovered from 100 g of faeces after culturing for 10 days following topical application with the equivalent of 200 litres per ha with either water ($H_2O$), effective micro-organism mixture (EM) or 20% liquid urea (N). Values are arithmetic means ± s.e.m**

**5.2 *In vitro* larvae culture when topically applied and incubated with and without cover**

Mean total number of larvae recovered per g faeces are given in Figure 5.2. Compared with the water with plastic bag treatment, egg hatching in the water without plastic bag treatment was reduced by 93% (P=0.008), by 100% for the urea without plastic bag (P=0.001) and 92% for urea with plastic bag (P=0.008). Within urea treatment there was no difference in the reduction in egg hatching between treatments that were with and without a plastic bag cover (P=0.42).

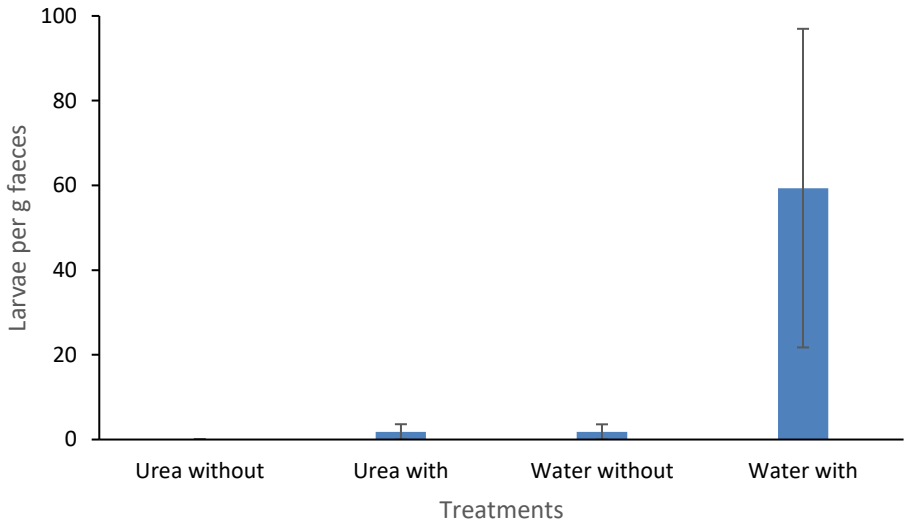


**Figure 5.2 Number of $L_3$ larvae recovered per g of faeces after culturing for 10 days at the equivalent of 2 ml of water ($H_2O$) and liquid urea solution (N) with and without a plastic bag covering the trays during culture. Values are arithmetic means ± s.e.m**

# Chapter 6 Discussion

The aim of the study was to extend the *in vitro* laboratory results of urea and effective microorganism performed by Cairns *et al.* (*2017*) and Lewis (2013) in the field, by providing a parasitic advantage on egg hatching while outside of it hosts. Furthermore, variables such as pasture herbage mass (pre-and post-grazing), faecal egg count and live weight gains were used as indicators post treatment, following topical application of urea (N) and effective micro-organism (EM) solutions applied to two days-old faeces to interrupt the parasite lifecycle. Moreover, the objective of the study was to determine the effectiveness of either treatment in reducing larval challenge on pasture and breaking the parasite lifecycle in grazing lambs.

Overall, there was little evidence to support the notion that application of either liquid urea solution (N) or EM influenced nematode development in the field (Figures 4.4-4.5). From all the variables that were used, there were no significant differences between treatments. A noticeable difference in the pasture larval contamination (Figures 4.4-4.5) shows little evidence that larval contamination was reduced for either *Strongyle* and *Nematodirus* specie for the liquid urea solution (N) treatment until day 17 when an increased presence of *Nematodirus* was observed. Removal of this outlier time point in the EM and urea (N) treatment is justified based on previous studies (Couvillion, 1993; Crofton, 1954; Donald, 1967; Taylor, 1939) who reported bias and incorrect sampling techniques can affect the result especially when samples are taken too close to the faecal mass. Despite, the removal of the outlier days on day 10 and 17 for EM and urea (N) respectively, the cumulative larval intake still showed no difference between treatments (Figures 4.7-4.9).

Overall, based on the pre- and post-grazing pasture herbage mass (Figures 4.1-4.3) and the cumulative larval intake (Figures 4.7-4.9), it was surprising to see there was no effect on FEC / estimated egg production (Figures 4.10-4.13) and lamb performance (Figure 4.14). Possible reasons may be the type of sampling method, as pasture larval are known to be variable. In the study, the method of herbage collection was the cutting of herbage from the base of the root which include stems and leaves. Previous studies (Vegora, 1960; Vlassoff, 1982) reported larvae migrate in soil to escape the heat from solar radiation (Sturrock, 1965) with 85% of *Trichostrongyle* $L_3$ larvae were found at depths of 5 cm in soil (Amaradasa *et al*., 2010) and 66.25% of *H. contortus* $L_3$ larvae were present at 2.5 cm herbage height, which then decrease to 20.66% at 5 cm herbage height. Other factors such as; the selective ability of sheep during grazing, the varying climatic conditions during the summer, the distribution of $L_3$ larvae on the grass sward and the over grazing of the pasture below the 2 cm herbage heights are the possible reasons. Therefore, taking into consideration all these variables, there is a possibility that

the cumulative larval intake may have been lower than the estimated numbers. In addition, the number of larvae present is dependent on the efficiency of the technique and the quantity of larvae recovered in the sediment (Donald, 1967).

Moreover, in all the treatment and replicate plots, all lambs grazed below the 2 cm herbage heights throughout each grazing rotation, suggesting overgrazing of pasture can exposed eggs and larvae which is deleterious to larval survival (Shorb, 1943; Stewart and Douglas, 1938) with little development occurring above 35°C (Familton and McAnulty, 1997 ; Silverman and Campbell, 1959 ; Vlassoff, 1982). Unfortunately, due to reduced pasture mass, rotation three could not have occurred to determine exactly how much of an influence each treatment has on reducing larval challenge on pasture. Therefore, it would be worthwhile to have a larger pasture for further investigations.

For FEC (Figures 4.10-4.13), there were fewer lambs emitting higher FEC within each treatment and replicate groups after drenching and continued throughout the trial, despite the addition of monospecificaly infected sheep faeces that was averaging 800 (epg). This therefore reflects low establishment of parasite, which may have been influenced by immunity due to the age of the lambs, which were five months old at the start of the trial and continued throughout the trial up to 7 to 8 months old. Therefore, further research is warranted in younger lambs 3-4 months old that have not acquired immunity to GIN infections. Previous studies of Tetley (1934) found *T. colubriformis* are threats to young lambs 5-6 months old and are seldom seen in lambs 6-9 months of age, while *Nematodirus* infection may be present in young lambs up to 6 months-of-age (Charleston, 1982; Tetley, 1935). In the study, the lambs that were emitting greater FEC had the greatest weight gain, suggesting that infection did not impact lamb's performance. Additionally, Greer and Sykes (2012) identified FEC does not provide a reliable indicator to the cost of parasitism as resilient animals may maintain performance while having high FEC. Moreover, Coop *et al*. (1982) reported lambs receiving 1000, 3000 and 5000 *T. colubriformis* $L_3$ larvae per day had live weight gains that were 90%, 75% and 53% respectively of the uninfected control. In this regard, based on the numbers of FEC (epg) that each lamb was emitting, it was not enough to create an impact on estimated egg production, pasture larval contamination, cumulative larval intake and lamb performance. Despite this, there are other possible factors that can impact FEC (epg) such as the infra-population mechanisms of GIN. Previous studies of Mupeyo *et al*. (2011) reported that the fecundity per female decreases as the number of worms increases (Bishop and Stear, 2000) which may restrict the ability of FEC to reflect the number of females present in a host.

Other noticeable effects were the considerable variability between replicates even on the same day which did not help with providing a consistent difference. In addition, the trial design was such that

variations in weather over a 16-day period could be observed. However, there were no apparent association between weather events (data not shown), but greatest variation occurred within replicates at the same treatment time. It is possible variation in treatment may have occurred by spraying of treatments which was carried out by hand. Therefore, from the time the treatment was applied to the pasture and the first grazing rotation of 16 days, larvae should have been developed and variability should have been reduced. Additionally, when comparing the results from the field and that of the *in vitro*, the lack of effect *in situ* was surprising, as the *in vitro* results suggested a very potent effect of urea (N) on egg development. The reason for the disparity is not clear, but the possibility of an artificial high ammonia concentration *in vitro* was ruled out by the plastic bag experiment (Figures 5.1- 5.2). In this regard, the design of the field study may have been a factor since treatment was only applied every second day, which may have provided an opportunity for half of the eggs to have developed, thus reducing the chances of seeing a difference. In addition, there is also the possibility that urea (N) influences egg development which may be sensitive to temperature fluctuations and environment conditions based on the presence of larvae on pasture sometime. Previous *in vitro* studies on urea (N) (Fidjeland *et al*., 2015; Pecson *et al*., 2007; Pecson and Nelson, 2005) who reported egg hatching decrease with time, pH, temperature and ammonia concentration.

On the other hand, there are other factors that could have influenced egg development such as the amount of urea (N) that penetrated the faecal mass which may not have being sufficient when dealing with faeces of different moisture or different surface area. It appears the 12-hour irrigation application that was applied to the field may have increased the faecal moisture, thus allowing the development of the *Nematodirus* population from exposed chilling, despite the pasture being new and no presence of eggs were detected in the seeding animals, or it may have stimulated the hatching of an existing population which seems unlikely but possible. Previous studies (Oliver *et al*., 2016) reported that it will take up to two years for one generation or more to be exposed to chilling with a maximum of 800-1000 chill units for hatching to occur. Further investigation on the amount of irrigation water to applied to pasture is worthwhile.

It is clear the effect of treatment on *strongyles* was confounded in the study due to the unexpected challenge of *Nematodirus*. If the *Nematodirus* was present in the soil it appears treatment stimulated their hatching. Moreover, recent studies of Bennett (2017) reported at the rates of N application used *in vitro Nematodirus* development was increased, although still reduced development at higher rates of N. Ultimately, it remains possible the treatment rates applied here may have inadvertently provided a treatment which favoured and encouraged *Nematodirus* development. Further investigations on the application rate required to have an effect of N in the field may be worthwhile.

The EM treatment appeared to favour larval development (Figures 4.4-4.5) with the effect in the field being consistent with the *in vitro* results which shows a 2.5-fold increase in egg hatching. This was surprising given the effect of EM and acid solutions on egg hatching. The findings differed from Lewis, (2013) who found 95% reduction in egg hatching when EM was autoclaved in lactic acid. Further, Cairns *et al*. (2017) found little or no egg hatching of *T. colubriformis* when pH was less than 5, while Dick and Leland (1973) reported no development of *Cooperia punctata* occurred when the acid pH was 6.4 to 6.9. The cause of this is uncertain but could be an area of fruitful research if treatments could be designed to stimulate larval development at times of the year when either survival may be low, or they could be grazed by non-susceptible stock to effectively reduce contamination. Similar approaches may also be useful in relation to the earlier mentioned apparent increase in *Nematodirus* larvae in the urea treatments. If this concentration of urea stimulates egg hatching for this species, then some control may be afforded as to when the contamination is present on pasture.

# Chapter 7 Conclusion/ Recommendation

Overall, the possibility of liquid urea solution (N) to break the parasite lifecycle needs further investigation despite *in vitro* result showing a very potent effect on egg development, with a reduction of 98 %. However, when the field results were compared with the plastic bag experiment, there was little development in egg hatching observed. Therefore, the possibility of an artificial high ammonia concentration was ruled out as the disparity is not clearly understood, therefore further investigation is worthwhile. For the design of the field, the two-day rotation may have provided an opportunity for half of the eggs to develop due to environmental conditions, and therefore suggests further amendments to the design that may not encourage egg development. Furthermore, the method of application of treatments using hand sprayer may allow the possibility of wind dispersing the treatments from one area to the next which may cause variation with the results. Therefore, for better dispersal it may be worthwhile to use a sprayer mounted to a tractor for evenly dispersal across treatment plots. Further, when comparing field with the lab, the lab provides a constant temperature which eliminates variation, and there is a need for further research on the dose rate of urea (N) in the field, since urea (N) may have inadvertently encourage *Nematodirus* development. On the other hand, chilling may provide the opportunity for *Nematodirus* larvae to emerge despite the pasture anticipated to be clean from GIN due to previous use. Therefore, further investigation is worthwhile on the rate per ha of irrigation water to applied to the field to prevent increased hatching from chilling.

Despite no significant difference between treatments, there is a possibility that the lambs used in the trial may have acquired immunity due to their age which was five months at the start of the trial and continued up to 7-8 month of age. Therefore, based on the variables and the amount of FEC (epg) produced, the values were low and could not have impacted lamb's performance, despite the heavier lambs were the ones with the greatest FEC. Further investigation is worthwhile on younger lambs 3-4 months old that have not acquired immunity.

Furthermore, for the EM treatment, the *in vitro* results showed a 2.5-fold increase in egg hatching, which did not different from the field, despite EM being acidic. This is a cause of concern, but it remains possible to use this as a tool to control when contamination is present, if appropriate steps to remove contamination can be taken.

# Appendix A

## 7.3 Rotation grazing in 10 x 10-meter plot

Before grazing 10mx10m treatment plots

During grazing 10mx10m treatment plots

After grazing section of 10 mx10m treatment plots

# References


Alderman, G., Cottrill, B., Esain Escobar, J., PHILLIPS, C., Melo, O. E., Boetto, G. C., . . . Domínguez Vivancos, A. (1993). Energy and protein requirements of rumiants: and advisory manual prepared by the AFRC Technical Committee on Responses to Nutrients: Agricultural and Food Research Council.

Amaradasa, B. S., Lane, R. A., & Manage, A. (2010). Vertical migration of Haemonchus contortus infective larvae on Cynodon dactylon and Paspalum notatum pastures in response to climatic conditions. Veterinary Parasitology*, 170*, 78-87.

Andersen, F. L., & Levine, N. D. (1968). Effect of desiccation on survival of the free-living stages of *Trichostrongylus colubriformis*. The Journal of Parasitology, 117-128.

Ashad, F., Anisuzzaman, M., Begum, N., Dey, A., & Mondal, M. (2013). Factors affecting the development and hatching of eggs and the survival of infective larvae of Haemonchus contortus in laboratory condition. Progressive Agriculture*,* 22, 75-83.

Bairden, K., Armour, J., & Duncan, J. L. (1995). A 4-year study of the effectiveness of alternate grazing of cattle and sheep in the control of bovine parasitic gastro-enteritis. Veterinary Parasitology, 60, 119-132.

Bishop, S. C., & Stear, M. J. (2000). The use of a gamma-type function to assess the relationship between the number of adult Teladorsagia circumcincta and total egg output. Parasitology, 121, 435-440.

Boag, B., & Thomas, R. J. (1985). The Effect of Temperature on the Survival of Infective Larvae of Nematodes. The Journal of Parasitology, 71, 383-384.

Brunsdon, R. (1960). Studies on the epizootiology of Nematodirus infestation in sheep in New Zealand. New Zealand Journal of Agricultural Research, 3, 772-778.

Brunsdon, R. (1963). Studies on the seasonal availability of the infective stages of Nematodirus filicollis and N. spathiger to sheep in New Zealand. New Zealand Journal of Agricultural Research, 6, 253-264.

Brunsdon, R. (1971). The post-parturient rise in the faecal nematode egg count of ewes: some host-parasite relationships. New Zealand Veterinary Journal, 19, 100-107.

Brunsdon, R. V. (1980). Principles of helminth control. Veterinary Parasitology, 6,185-215.

Cabaret, J., & Berrag, B. (2004). Faecal egg count reduction test for assessing anthelmintic efficacy: average versus individually based estimations. Veterinary Parasitology, 121, 105-113.

Cairns, J., McAnulty, R. W., & Greer, A. W., &. (2017). Potential anthelmintic properties of urea: New Zealand Society of Animal Production.

Chamberlain, T., Daly, M., & Merfield, C. (1997). Utilisation of effective microorganisms commercial organic agriculture: a case study from New Zealand Symposium conducted at the meeting of the 5th International Nature Farming Conference

Charleston, W. (1982). An introduction to gastrointestinal nematode parasites of sheep and cattle in New Zealand Symposium conducted at the meeting of the Control of Internal Parasites in Sheep: Animal Industries Workshop. Lincoln College, Veterinary Services Council, Ministry of Agriculture and Fisheries.

Chaves, A. V., Burke, J. L., Waghorn, G. C., & Brookes, I. M. (2006). Digestion kinetics of leaf, stem and inflorescence from five species of mature grasses. Journal of the Science of Food and Agriculture, 86, 816-825.

Coop, R., Sykes, A., & Angus, K. (1982). The effect of three levels of intake of Ostertagia circumcincta larvae on growth rate, food intake and body composition of growing lambs. The Journal of Agricultural Science, 98, 247-255.

Couvillion, C. E. (1993). Special Issue: Ostertagia Estimation of the numbers of trichostrongylid larvae on pastures. Veterinary Parasitology, 46, 197-203.

Coyne, M., Smith, G., & Johnstone, C. (1991). Fecundity of gastrointestinal trichostrongylid nematodes of sheep in the field. American Journal of Veterinary Research, 52, 1182-1188.

Crofton, H. (1954). The ecology of the immature phases of trichostrongyle parasites V. The estimation of pasture infestation. Parasitology, 44, 313-324.

Crofton, H. (1957). Nematode parasite populations in sheep on lowland farms III. The seasonal incidence of species. Parasitology, 47, 304-318.

Crofton, H. (1958). Nematode parasite populations in sheep on lowland farms VI. Sheep behaviour and nematode infections. Parasitology, 48, 251-260.

Crofton, H. D. (1948). The ecology of immature phases of Trichostrongyle nematodes: I. The vertical distribution of infective larvae of Trichostrongylus retortaeformis in relation to their habitat. Parasitology, 39, 17-25.

Dick, J. W., & Leland, S. E. (1973). The Influence of pH on the In vitro Development of Cooperia punctata (Ransom, 1907). The Journal of Parasitology, 59, 770-775.

Donald, A. (1967). A technique for the recovery of strongyloid infective larvae from small sample units of pasture. Journal of Helminthology, 41, 1-10.

Eriksen, L., Andreasen, P., & Ilsoe, B. (1996). Inactivation of Ascaris suum eggs during storage in lime treated sewage sludge. Water Research, 30, 1026-1029.

Familton, A., & McAnulty, R. (1997). Life cycles and development of nematode parasites of ruminants. In G.K.Barrell( (editor), Sustainable control of internal parasites in ruminants : Animal industries workshop,June 1997 (p 67-80). Christchurch : Lincoln University.

Fidjeland, J., Nordin, A., Pecson, B. M., Nelson, K. L., & Vinneras, B. (2015). Modeling the inactivation of ascaris eggs as a function of ammonia concentration and temperature. Water Research (Oxford), 83, 153-160.

Fidjeland, J., Nordin, A., Pecson, B. M., Nelson, K. L., & Vinneras, B. (2016). Modeling the inactivation of ascaris eggs as a function of ammonia concentration and temperature . Water Research 153, 384.

Flota-Bañuelos, C., Martínez, I., López-Collado, J., Vargas Mendoza, M., González Hernández, H., & Fajersson, P. (2013). Spatio-temporal pattern of larvae and eggs of gastrointestinal nematodes in cattle pastures in Veracruz, Mexico. Revista de biologia tropical, 61, 1747-1758.

Freitag, D. G., & Meihoefer, H. (2000). The use of Effective Microorganisms (EM) in organic waste management. Effective Microorganisms@ emtrading. com.

Gazda, T. L., Piazzetta, R. G., Dittrich, J. R., Monteiro, A. L. G., & Thomaz-Soccol, V. (2009). Distribution of nematode larvae of sheep in tropical pasture plants. Small Ruminant Research, 82, 94-98.

Ghiglietti, R., Genchi, C., Di Matteo, L., Calcaterra, E., & Colombi, A. (1997). Survival of Ascaris suum eggs in ammonia-treated wastewater sludges. Bioresource Technology, 59, 195-198.

Goldberg, A. (1968). Development and survival on pasture of gastrointestinal nematode parasites of cattle. The Journal of Parasitology, 856-862.

Gordon, H. M. (1948). The epidemiology of parasitic diseases, with special reference to studies with nematode parasites of sheep Australian Veterinary Journal, 24, 17-45.

Gordon, H. M. (1967). The diagnosis of helminthosis in sheep. Veterinary Medicine Revision, Leverkusen, 140-168.

Greer, A. W., & Sykes, A. R. (2012). Are faecal egg counts approaching their "sell-by" date? Proceedings of the New Zealand Society of Animal Production, 72, 199-204.

Gruner, L., & Sauve, C. (1982). The distribution of trichostrongyle infective larvae on pasture and grazing behaviour in calves. Veterinary Parasitology, 11, 203-213.

Higa, T., & Wididana, G. (1991). Changes in the soil microflora induced by effective microorganisms Symposium conducted at the meeting of the Proceedings of the First International Conference on Kyusei Nature Farming. US Department of Agriculture, Washington, DC, USA

Holasová, E., Pavlásek, I., & Kotrlá, B. (1988). Effects of basic abiotic factors on development of the eggs and on survival of the infective larvae of common helminths of sheep in the external environment. Acta Veterinaria Brno, 57, 153-168.

Howell, J., Luginbuhl, J.-M., Grice, M., Anderson, K. L., Arasu, P., & Flowers, J. R. (1999). Control of gastrointestinal parasite larvae of ruminant using nitrogen fertilizer, limestone and sodium hypochlorite solutions. Small Ruminant Research, 32, 197-204.

Jackson, F., Bartley, D., Bartley, Y., & Kenyon, F. (2009). Worm control in sheep in the future. Small Ruminant Research, 86, 40-45.

John, A., & Ulyatt, M. (1987). Importance of dry matter content to voluntary intake of fresh grass forages Symposium conducted at the meeting of the Proceeding New Zealand Society of Animal Production

Kaplan, R. M. (2006). Update on parasite control in small ruminants 2006: addressing the challenges posed by multiple–drug resistant worms. Proceeding American Association Bovine Practicals, Saint Paul, Minnesota, 39, 1-16.

Kaplan, R. M., & Vidyashankar, A. N. (2012). An inconvenient truth: Global worming and Anthelmintic Resistance. Veterinary Parasitology, 186, 70-78.

Katakam, K. K., Mejer, H., Dalsgaard, A., Kyvsgaard, N. C., & Thamsborg, S. M. (2014). Survival of Ascaris suum and Ascaridia galli eggs in liquid manure at different ammonia concentrations and temperatures. Veterinary Parasitology, 204, 249-257.

Kato, S., Reimers, R. S., Fogarty, E. A., & Bowman, D. D. (2001). Effect of Aerobic Digestion, Anaerobic Digestion, and Ammonia on the Viability of Oocysts of Cryptosporidium parvum and the Eggs of Ascaris suum in Sewage Sludges. Proceedings of the Water Environment Federation, 2001, 1179-1191.

Kenyon, F., Greer, A. W., Coles, G. C., Cringoli, G., Papadopoulos, E., Cabaret, J., . . . Jackson, F. (2009). The role of targeted selective treatments in the development of refugia-based approaches to the control of gastrointestinal nematodes of small ruminants. Veterinary Parasitology, 164, 3-11.

Khadijah, S., Kahn, L. P., Walkden-Brown, S. W., Bailey, J. N., & Bowers, S. F. (2013). Soil moisture modulates the effects of the timing and amount of rainfall on faecal moisture and development of Haemonchus contortus and Trichostrongylus colubriformis to infective third stage larvae. Veterinary Parasitology, 196, 347-357.

Khatun, F., Begum, N., Akter, S., & Mondal, M. M. H. (2013). In vitro study of environmental and nutritional factors on the hatching and development of eggs of Haemonchus contortus. Bangladesh Veterinarian, 30, 1-9.

Leathwick, D., Barlow, N., & Vlassoff, A. (1992). A model for nematodiasis in New Zealand lambs. International Journal for Parasitology, 22, 789-799.

Leathwick, D., Waghorn, T., Miller, C., Candy, P., & Oliver, A.-M. (2012). Managing anthelmintic resistance–use of a combination anthelmintic and leaving some lambs untreated to slow the development of resistance to ivermectin. Veterinary Parasitology, 187, 285-294.

Lettini, S., & Sukhdeo, M. V. K. (2006). Anhydrobiosis increases survival of trichostrongyle nematodes. Journal of Parasitology, 92, 1002-1009.

Litherland AJ, D. D. L. (2008). Quantifying parasitic nematode larvae on pasture: Proceedings of the New Zealand Society of Animal Production.

Lokare, C. (2007). Effective Microorganisms: myth or reality? Review Peru Biology, 14, 315-319.

M.A.F.F. (1977). Manual of veterinary parasitological laboratory techniques. Retrieved from <Go to ISI>://CABI:19772297994

Martin, R. R., Beveridge, I., Pullman, A. L., & Brown, T. H. (1990). A modified technique for the estimation of the number of infective nematode larvae present on pasture, and its application in the field under South Australian conditions. Veterinary Parasitology, 37, 133-143.

Maya, C., Torner-Morales, F. J., Lucario, E. S., Hernandez, E., & Jimenez, B. (2012). Viability of six species of larval and non-larval helminth eggs for different conditions of temperature, pH and dryness. Water Research (Oxford), 46, 4770-4782.

McKenna, P. (1977). The diagnosis of gastrointestinal nematode parasitism in ruminants and investigating anthelmintic resistance. Sustainable Control of Internal Parasites in Ruminants, 93-106.

McKenna, P. (1997). The diagnosis of gastrointestinal nematode parasitism in ruminants and investigating anthelmintic resistance. In B. GK (Chair), Symposium conducted at the meeting of the Sustainable control of internal parasites in ruminants: Animal Industries Workshop

McKenna, P. B. (1981). The diagnostic value and interpretation of faecal egg counts in sheep. New Zealand Veterinary Journal, 29, 129-132.

Miller, C. M., Waghorn, T. S., Leathwick, D. M., Candy, P. M., Oliver, A. M. B., & Watson, T. G. (2012). The production cost of anthelmintic resistance in lambs. Veterinary Parasitology, 186, 376-381.

Misra, S., & Ruprah, N. (1973). Effects of temperature, relative humidity and pH on Haemonchus contortus eggs. Indian Veterinary Journal.

Morgan, E. (2011). The influence of water on the migration of infective trichostrongyloid larvae onto grass. Parasitology, 138, 780-788.

Morley, F. H. W., & Donald, A. D. (1980). Farm management and systems of helminth control. Veterinary Parasitology, 6, 105-134.

Moss, R., & Bray, A. (2006). Effect of sward density and size of faecal deposit on the development and persistence of third-stage Trichostrongylid larvae of sheep. New Zealand Journal of Agricultural Research, 49, 475-481.

Mupeyo, B., Barry, T. N., Pomroy, W. E., Ramírez-Restrepo, C. A., López-Villalobos, N., & Pernthaner, A. (2011). Effects of feeding willow (Salix spp.) upon death of established parasites and parasite fecundity. Animal Feed Science and Technology, 164, 8-20.

Namsivayam, S. K. R., Narendrakumar, G., & Kumar, J. A. (2011). Evaluation of Effective Microorganism (EM) for treatment of domestic sewage. Journal of Experimental Sciences, 2.

O'Connor, L. J., Kahn, L. P., & Walkden-Brown, S. W. (2007). Moisture requirements for the free-living development of Haemonchus contortus: Quantitative and temporal effects under conditions of low evaporation. Veterinary Parasitology, 150, 128-138.

Oliver, A. M. B., Pomroy, W. E., Ganesh, S., & Leathwick, D. M. (2016). Chilling requirements for hatching of a New Zealand isolate of Nematodirus filicollis. Veterinary Parasitology, 226, 17-21.
Pandey, V., Chaer, A., & Dakkak, A. (1989). Effect of temperature on development of the free-living stages of Ostertagia circumcincta. Veterinary parasitology, 32, 193-197.

Pecson, B. M., Barrios, J. A., Jimenez, B. E., & Nelson, K. L. (2007). The effects of temperature, pH, and ammonia concentration on the inactivation of Ascaris eggs in sewage sludge. Water Research (Oxford), 41, 2893-2902.

Pecson, B. M., & Nelson, K. L. (2005). Inactivation of Ascaris suum eggs by ammonia. Environmental Science & Technology, 39, 7909-7914.

Reimers, R. S., McDonell, D. B., Little, M. D., Bowman, D. D., Englande, A. J., Jr., & Henriques, W. D. (1986). Effectiveness of wastewater sludge treatment processes to inactivate parasites. Water Science and Technology, 18, 397-404

Rocha, R. A. d., Bricarello, P. A., Rocha, G. P. d., & Amarante, A. F. T. d. (2014). Retrieval of Trichostrongylus colubriformis infective larvae from grass contaminated in winter and in spring. Revista Brasileira de Parasitologia Veterinária, 23, 463-472.

Roeber, F., Jex, A. R., & Gasser, R. B. (2013). Impact of gastrointestinal parasitic nematodes of sheep, and the role of advanced molecular tools for exploring epidemiology and drug resistance-an Australian perspective. Parasites & Vectors, 6,1.

Schwartz, B., & Price, E. (2011). The Effect of UV Radiation on Survival of the Free-Living Stages of Haemonchus contortus.

Shorb, D. A. (1943). Survival on Grass Plots of Eggs and Preinfective Larvae of the Common Sheep Stomach Worm, Haemonchus contortus. The Journal of Parasitology, 29, 284-289.

Silangwa, S. M., & Todd, A. C. (1964). Vertical Migration of Trichostrongylid Larvae on Grasses. The Journal of Parasitology, 50, 278-285.

Silverman, P. H., & Campbell, J. A. (1959). Studies on parasitic worms of sheep in Scotland. Parasitology, 49, 23-38.

Smeal, M. G., & Hendy, G. A. (2009). A Technique for the Recovery of Strongyloid Infective Larvae from Pasture. Journal of Helminthology, 46, 201-211.

Southcott, W. H., & Barger, I. A. (1975). Control of nematode parasites by grazing management—II. Decontamination of sheep and cattle pastures by varying periods of grazing with the alternate host. International Journal for Parasitology, 5, 45-48.

Steel, J., Symons, L., & Jones, W. (1980). Effects of level of larval intake on the productivity and physiological and metabolic responses of lambs infected with Trichostrongylus colubriformis. Crop and Pasture Science, 31, 821-838.

Stewart, M. A., & Douglas, J. (1938). Studies on the bionomics of Trichostrongylus axei (Cobbold) and its seasonal incidence on irrigated pastures. Parasitology, 30, 477-490.

Stringfellow, F. (1986). Cultivation of Haemonchus contortus (Nematoda: Trichostrongylidae) from infective larvae to the adult male and the egg-laying female. The Journal of Parasitology, 339-345.

Stromberg, B. E. (1997). Environmental factors influencing transmission. Veterinary Parasitology, 72, 247-264.

Sturrock, R. F. (1965). The control of trichostrongyle larvae (Nematoda) by fumigation in relation to their bionomics. Parasitology, 55, 29-44.

Symons, L., Steel, J., & Jones, W. (1981). Effects of level of larval intake on the productivity and physiological and metabolic responses of lambs infected with Ostertagia circumcincta. Crop and Pasture Science, 32, 139-148.

Szymanski, N., & Patterson, R. A. (2003). Effective microorganisms (EM) and wastewater systems. Future Directions For On-Site Systems: Best Management Practice Proceedings of On-Site, 3, 347-354.

Taylor, E. (1939). Technique for the estimation of pasture infestation by strongyloid larvae. Parasitology, 31, 473-478.

Taylor, M., Learmount, J., Lunn, E., Morgan, C., & Craig, B. (2009). Multiple resistance to anthelmintics in sheep nematodes and comparison of methods used for their detection. Small Ruminant Research, 86, 67-70.

Tetley, J. (1934). The nematodes of sheep in Manawatu district, New Zealand. Journal of Helminthology, 12, 183-196.

Tetley, J. (1935). Ecological studies on Nematodirus species in sheep in Manawatu District, New Zealand. Journal of Helminthology, 13, 41-58.

Van Dijk, J., De Louw, M., Kalis, L., & Morgan, E. (2009). Ultraviolet light increases mortality of nematode larvae and can explain patterns of larval availability at pasture. International Journal for Parasitology, 39, 1151-1156.

van Dijk, J., & Morgan, E. R. (2008). The influence of temperature on the development, hatching and survival of Nematodirus battus larvae. Parasitology, 135, 269-283.

Van Houtert, M. (1997). Effects of diet on gastrointestinal nematode infection in ruminants. In G.K.Barrell (editor), Sustainable Control of Internal Parasites in Ruminants, (p 183-192).

Veglia, F., &. (1916). The anatomy and life-history of the Haemonchus contortus (Rud). Third and Fourth Reports of the Director of Veterinary Research: Agriculture.

Vegora, H. (1960). The effect of forage height on the development of cattle nematode larvae. Journal of Parasitology, 46, 39-40.

Verschave, S. H., Levecke, B., Duchateau, L., Vercruysse, J., & Charlier, J. (2015). Measuring larval nematode contamination on cattle pastures: Comparing two herbage sampling methods. Veterinary Parasitology, 210, 159-166.

Vlassoff, A. (1973). Seasonal incidence of infective trichostrongyle larvae on pasture grazed by lambs. New Zealand Journal of Experimental Agriculture, 1, 293-301.

Vlassoff, A. (1976). Seasonal incidence of infective trichostrongyle larvae on pasture: the contribution of the ewe and the role of the residual pasture infestation as sources of infection to the lamb. New Zealand Journal of Experimental Agriculture, 4, 281-284.

Vlassoff, A. (1982). Biology and population dynamics of the free-living stages of gastrointestinal nematodes of sheep. ROSS, AD Control of internal parasites in sheep.New Zealand:Lincoln College, 11-20.

Vlassoff, A., Leathwick, D. M., & Heath, A. C. G. (2001). The epidemiology of nematode infections of sheep. New Zealand Veterinary Journal, 49, 213-221.

Waller, P. J. (2003). Global perspectives on nematode parasite control in ruminant livestock: the need to adopt alternatives to chemotherapy, with emphasis on biological control. Animal Health Research Reviews, 4, 35-44.

Waller, P. J. (2006). Sustainable nematode parasite control strategies for ruminant livestock by grazing management and biological control. Animal Feed Science and Technology, 126, 277-289.

Wang, T., van Wyk, J. A., Morrison, A., & Morgan, E. R. (2014). Moisture requirements for the migration of Haemonchus contortus third stage larvae out of faeces. Veterinary Parasitology, 204, 258-264.

Wei-jiong, L., Yong-zhen, N., Umemura, H., & Qin-long, S. (1996). Effect of EM on Crops and Animal Husbandry in China Symposium conducted at the meeting of the Proceeding third Conference on Effective Microorganism. INFRC Japan and APNAN Thailand.

Wood, M. T., Miles, R., & Tabora, P. (1997). EM fermented plant extract and EM5 for controlling pickleworm (Diaphania nitidalis) in organic cucumber. School of Natural Resources, University of Missouri, USA and EARTH College, Limon, Costa Rica.

Young, R. t., & Trajstman, A. (1980). A rapid techinque for the recovery of strongyloid infective larvae from pasture and soil samples. Parasitology, 80, 425-431.